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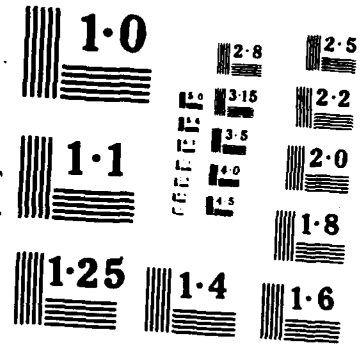
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ANALYSIS OF TRICHOTHECENE MYCOTOXINS BY COMBINED HPLC/MS

ANNUAL SUMMARY REPORT

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The development of HPLC/MS assays for the analysis of selected trichothecene mycotoxins in urine is the goal of this program. T-2 toxin has been the initial target of this investigation. Three important issues have been addressed before proceeding with the actual HPLC/MS experiments: the synthesis of isotopically labelled internal standards, efficient recovery of the target compounds from the urine matrix, and determination of HPLC conditions for their separation.

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19. ABSTRACT (continued)

Trideuteriated T-2 toxin was synthesized by peracetylation of HT-2 with hexadeuterioacetic anhydride, followed by selective hydrolysis of the C-3 acetate. The labelled T-2 was separated and purified chromatographically with a final yield of 62%.

Tritium labelled T-2 was used to establish the optimum conditions for recovery of the analyte from urine. Ethyl acetate was found to recover 99% of the T-2 at levels as low as 1ng/mL of urine. A more generalized procedure which takes into consideration the concurrent recovery of polar metabolites of T-2 from urine involves the use of n-butanol as an extraction solvent from a salted out solution. Nearly 80% recovery of all hydrolysis products is indicated under these conditions. The stability of T-2 toxin in alcoholic-aqueous buffer solution of pH = 7.0 was determined. The half-life of T-2 is estimated at 50-60 days.

A precolumn system has been designed which provides for an on-line isolation of the analytes prior to injection into the analytical HPLC column. Efficient analysis of urinary samples of T-2 and HT-2 without prior elaborate conventional extractions appear to be possible via this approach. The optimal separation conditions for T-2, HT-2, DON, DAS, T-2 tetraol, Nivalenol and Verrucarol have been established. The k' values of all the compounds have been determined and a gradient in the range between 5% and 50% acetonitrile in water should provide for complete separation of the analytes.

Mass spectra of the compounds were recorded with a Finnigan 4000 MS using the moving belt interface under chemical ionization conditions with ammonia as reagent gas. For the most part the spectra were dominated by $[M+NH_4]^+$ molecular adduct ions. For T-2 toxin, the detection limit was of the order of 1-5 ng "spotted" onto the belt. Significant improvement in sensitivity is expected with our newly acquired VG-7070SE mass spectrometer where the belt interface enters into the ion volume. The latter system will be employed on the forthcoming on-line HPLC/MS studies.

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FOREWORD

The objective of this program is the development of HPLC/MS assays for selected trichothecene mycotoxins of biological significance. Consistent with the agreement reached at the meeting of May 1985 between the principal investigators and the program monitors representing Ft. Detrick, we selected T-2 toxin as the first target of this investigation. The concurrent analysis of potential metabolites of T-2 has also been taken into consideration in the development of the HPLC/MS methodology. In line with this requirement, pertinent characteristics of the trichothecenes HT-2, DON, DAS, T-2 tetraol, Nivalenol and Verrucarol have also been examined in the course of this work.

Crucial to the development of an HPLC/MS assay are the availability of isotopically labeled internal standard(s) and an efficient recovery procedure to isolate the compound(s) of interest from the medium under consideration, in this case urine. Therefore, much preliminary work is needed before proceeding with actual HPLC/MS experiments. For the case of T-2 and its potential metabolites, the bulk of these preliminary experiments have been completed and we are now in the process of setting up the HPLC/MS analysis. Accordingly, our work to this date has focussed on the following four major areas:

1. The synthesis of deuterium labelled T-2

2. Optimization of procedures for isolation of T-2 and its potential metabolites from urine.

3. Establishment of HPLC conditions for separation of the target compounds, preferably in a single analysis.

4. Examination of the mass spectra of selected compounds using the moving belt sample inlet.

The synthesis of trideuteriated-T-2 was based on a peracetylation of HT-2 toxin with deuteriated acetic anhydride, followed by selective hydrolysis of the C-3 acetate.

Tritium labelled T-2 was used to optimize the conditions for recovery of the compound from urine. Recovery of 100% of the compound from urine was achieved. Radiolabelled T-2 was also used to assess the stability of T-2 against hydrolysis in alcoholic-aqueous buffer solutions. Good stability, with half-life of 50-60 days was found. Finally, extraction from salted-out urine into n-butanol was found to provide a system for recovery of approximately 80% of T-2 and its polar hydrolysis products which are also potential metabolites of T-2.

The chromatographic separation of T-2 and its potential metabolites requires use of gradient HPLC, if it is going to be carried out in a single analysis because of the wide range of polarities of the related compounds. Rather than use a trial and error approach, we have opted to determine systematically the k' values of the various compounds of interest in order to establish the optimum gradient conditions for analysis. This part of the work has now been completed. In addition, we have evaluated a precolumn injection and cleanup system which permits us to conduct the analysis of urine samples containing mycotoxins via a procedure of an on-line trapping and subsequent flushing of the analytes into the main analytical column for separation. The system has been tested thus far with urine samples spiked with T-2 and HT-2, and recoveries of more than 90% of the compounds was achieved. It was the intent of this program to streamline the procedures for analysis of trichothecenes by reducing the number of cleanup and other preliminary sample preparation steps. It appears that this precolumn enrichment approach may allow us to by-pass some of the conventional extraction steps and help achieve this goal.

Most of our work to this date has employed UV rather than MS detection in order to simplify and expedite the experimentation. Since our original goal was to transfer the technology and information derived from this program to the colleagues at Ft. Detrick, we plan to carry out the HPLC/MS on a VG-7070SE mass spectrometer, of the type available at Ft. Detrick.

This instrument has just been installed in our laboratory and we are now ready to proceed with this phase of the work. Some preliminary evaluation of the mass spectrometric characteristics of the compounds using the moving belt LC/MS inlet has been performed which shows that the toxins examined can be introduced into the MS without degradation. The detailed results of our studies thus far are discussed below.

1. THE SYNTHESIS OF d_3-T-2 TOXIN

Trichothecene mycotoxins are a group of sesquiterpenoid compounds produced by several genera of Fungi Imperfecti. Many trichothecenes are known and several of them are natural contaminants of cereal grains(1). Ingestion of the contaminated foodstuffs by humans or farm animals is known to cause a wide variety of toxicoses often leading to death(2). Trichothecenes have also recently been implicated as chemical warfare agents(3).

The determination of trichothecene mycotoxins has been a subject of considerable investigation in recent years(4). The methods used include TLC, GC, GC-MS, LC, and LC-MS(5). Many of these methods require extensive sample preparation to remove the matrix components. In addition, because many of the trichothecenes of interest are very polar and have no chromophoric group for UV or fluorescence detection, derivatization is often needed for quantitative determination(6).

In our analysis of trichothecene mycotoxins and their metabolites in biological fluids by combined HPLC-MS, we required a standard for quantitation. Many standards have been used in GC and MS analyses of trichothecenes including n-dotriacontane(7), known quantities of the analyte(8), and trichothecenes derivatized with deuteriated trimethylsilyl groups(9). However, the ideal internal standard would be a simple deuteriated sample of the trichothecene to be analyzed. It is therefore surprising that simple deuteriated trichothecenes are not readily available for use as internal standards(10).

There are different approaches to the synthesis of deuterium labelled analogs of trichothecenes, such as T-2 toxin. Previously mentioned was the formation of deuteriated TMS derivatives. The major disadvantage to this method is the widely differing chromatographic behavior between the internal standard and the analyte. Moreover, trimethylsilyl ethers are susceptible to hydrolysis and, in a mixture of TMS-d_0 and TMS-d_9 derivatives of an analyte, cross silylation which may lead to erroneous results is always a possibility. Another approach is to go through an oxidation-reduction

sequence as described in the synthesis of radiolabelled T-2 toxin(11). However, this approach is low-yielding and the resultant molecular ion in the mass spectrum of the standard would differ from that of the unlabelled compound by only one mass unit.

In our approach the trichothecene of interest, T-2 toxin, was labelled with deuteriated acetate. Investigations into the chemistry of trichothecenes have shown that the ester groups located in different areas of the molecule exhibit different hydrolysis rates(12). Thus several trichothecenes may be made available by carefully monitored hydrolysis of a per-esterified precursor.

For the synthesis of the title compound 3, we envisioned a two-step sequence (figure 1) involving peracetylation of HT-2 toxin 1 with deuterium labelled acetic anhydride followed by selective hydrolysis of the C-3 acetate. HT-2 toxin was chosen as the starting material for its ready availability. Acetylation of HT-2 toxin with acetic anhydride-d_6 in pyridine yielded quantitatively the peracetate 2 as evidenced by the incorporation of six deuterium atoms in the mass spectrum (figure 2). The peak of highest mass at m/z 430 in the EI mass spectrum of the labelled compound is a fragment ion produced by the loss of the isovaleryl group from the molecular ion. The fragmentation apparently does not involve the acetyl groups at the C-3 and C-4 positions, since the ion mass is shifted by six mass units from m/z 424 in the spectrum of the unlabelled compound to m/z 430 in the synthesized labelled analog. Chemical ionization (NH_3) of the labelled compound 2 yielded a molecular adduct ion $[M+NH_4]^+$ of the expected mass of 532. In addition, TLC data and the presence of only one acetate methyl and the shift of the C-3 and C-4 protons from 4.26 and 4.42 ppm to 5.21 and 5.95 ppm respectively in the NMR spectrum (figure 3) further support the formation of this compound. A portion of peracetate 2 was hydrolyzed in 1M ammonium hydroxide in methanol-water (4:1). After a reaction time of 21 hrs, TLC revealed that very little T-2 toxin 3 was produced. The peracetate 2 extensively hydrolyzed to the starting material, HT-2 toxin, and to more polar products. This result was surprising since Katsonis and Ellison(13), in their investigation of HT-2 toxin formation in liquid culture, prepared hexadeuterio-T-2 toxin in 23% overall yield by a

similar procedure. The hydrolysis of peracetate 2 was repeated with careful monitoring by TLC at half hour intervals. After 90 min, TLC revealed that the peracetate 2 was no longer present, and the major component was T-2 toxin 3 along with smaller amounts of HT-2 toxin. The ratio of T-2 toxin to HT-2 toxin in the crude reaction mixture was 4:1 as determined by respective signal heights of the H-2 and H-13 protons in the region 2.5-4.0 ppm of the NMR spectrum (figure 4). Thus, the overall yield of d_3-T-2 toxin synthesized by this method is nearly 80%, far better than the 23% overall yield reported by Katsonis and Ellison(13) for their d_6-analog. The d_3-T-2 toxin 3, purified by prep TLC, had a mass spectrum (figure 5) identical to the unlabelled compound with the exception of the higher mass peaks which differed by three mass units (m/z 385/382, 367/364, 307/304, 281/278). The isotopic purity for compounds 2 and 3 was determined by mass spectrometry to be greater than 99%.

Experimental

All chemicals were used as obtained from the manufacturer except where noted otherwise. Melting points were obtained on a Thomas Hoover melting point apparatus and are uncorrected. NMR spectra were recorded in deuteriochloroform using a Varian 300MHz spectrometer with tetramethylsilane as the internal reference. Mass spectra were obtained with a Finnigan Model 4000 spectrometer or with a Nuclide Model 1290-G mass spectrometer.

Hexadeuterioacetyl T-2 toxin, 2

HT-2 toxin (Myco Labs, 50 mg) was dissolved in freshly distilled pyridine (1 mL) and hexadeuterioacetic anhydride (1 mL) and allowed to react at room temperature for 24 hrs. Water was then added and the mixture was extracted with ethyl acetate. The organic layer was washed with 1N HCl, water and then dried over sodium sulfate. A pale yellow oil was obtained, 60 mg. NMR: δ 0.77(s, 3H, H-14), 0.98(d, 3H, J = 6Hz, H-4'), 0.99(d, 3H, J = 6Hz, H-4'), 1.77(s, 3H, H-16), 1.92(d, 1H, J = 15Hz, H-7a), 2.10(s, 3H, OAc), 2.39(dd, 1H, J = 5, 15Hz, H-7b), 2.85(d, 1H, J = 4Hz, H-13a), 3.10(d, 1H, J = 4Hz, H-13b), 3.89(d, 1H, J = 5Hz, H-2), 4.14(d, 1H, J = 12Hz, H-15a), 4.24(d, 1H, J = 6Hz, H-11), 4.38(d, 1H, J = 12Hz, H-15b), 5.21(dd, 1H,

J = 5, 4Hz, H-3), 5.32(d, 1H, J = 5Hz, H-8), 5.78(d, 1H, J = 6Hz, H-10), 5.95(d, 1H, J = 4Hz, H-4); mass spectrum m/z (rel. intensity) 430(8,M^+-C_4H_8CO), 412(37,M^+-C_4H_9COOH), 370(5), 353(5), 352(6), 326(6), 275(28), 227(44), 180(43), 121(47), 105(52), 85(44), 57(62,$C_4H_9^+$), 46(100,CD_3CO^+).

Trideuterio T-2 toxin, 3

Hexadeuterioacetyl T-2 toxin, 2, was dissolved in a solution (4 mL) of 1M ammonia in methanol-water (4:1). Aliquots of the reaction mixture were analyzed by TLC at 30 min intervals. The TLC plates (Analtech Uniplates silica gel GF) were developed in hexane; ethyl acetate (1:5) and visualized by dipping the plates in a solution of 3% p-nitrobenzyl pyridine in chloroform/carbon tetrachloride (2:3), heating for 30 min at 160°C and dipping in 10% solution of 1,6-hexanediamine in $CHCl_3$-CCl_4 (2:3). The trichothecenes appeared as blue spots on a white background. After 90 min of reaction time the reaction was quenched with 1N HCl and the mixture was extracted with ethyl acetate, washed with water, and dried over sodium sulfate. NMR and TLC analysis indicated that the crude mixture consisted of ~80% T-2 toxin, ~20% HT-2 toxin, and only a trace of starting material. The T-2 toxin residue was purified by TLC (Analtech Uniplates Silica Gel GF) using hexane: ethyl acetate (1:5). The zone corresponding to R_f 0.44 to 0.63 was scraped, and the silica was extracted with ethyl acetate, filtered, and dried (34 mg, 62% isolated yield) mp 144-146°. NMR δ 0.83 (s, 3H, H-14), 0.99 (d, 3H, J = 7Hz, H-4'), 1.00 (d, 3H, J = 7Hz, H-4'), 1.78(s, 3H, H-16), 1.94(d, 1H, J = 16 Hz, H-7a), 2.07(s, 3H, OAc), 2.43(dd, 1H, J = 16, 6 Hz, H-7b), 2.83(d, 1H, J = 4Hz, H-13a), 3.09(d, 1H, J = 4Hz, H-13b), 3.73 (d, 1H, J = 5Hz, H-2), 4.09(d, 1H, J = 14Hz, H-15a), 4.19(dd, 1H, J = 5, 4Hz, H-3), 4.32(d, 1H, J = 14Hz, H-15b), 4.38(d, 1H, J = 5Hz, H-11), 5.32 (d, 1H, J = 5Hz, H-8), 5.35(d, 1H, J = 4Hz, H-4), 5.84(d, 1H, J = 5Hz, H-10); mass spectrum m/z 385(1.1, M^+-C_4H_8CO), 367(3.3, M^+-C_4H_9COOH), 325(0.5), 308(2), 307(2), 281(4), 261(2), 205(6), 180(16), 121(51), 105(26), 85(33), 57(69, $C_4H_9^+$), 46(100, CD_3CO^+).

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2. RECOVERY STUDIES

a. Recovery of ^{3}H-T-2 Toxin from Urine

Several methods for the extraction of trichothecene mycotoxins are available(1). Because the matrices involved are generally moldy grains, the solvents most often used for the isolation of the trichothecenes have been mixtures of methanol, ethanol and acetonitrile with water.(2) Clearly, these solvents are not appropriate when the matrix is an aqueous solution, as is urine. We therefore studied the partition of a trichothecene, T-2 toxin, between various water-insoluble organic solvents and water, urine, and saturated sodium chloride solution. In a typical experiment, ^{3}H-T-2 toxin (0.5 µCi in 10 µL solvent) was partitioned between the organic solvent (1 mL) and aqueous solvent (1 mL). The layers were separated and 0.5 mL of each layer was counted for 2 minutes in scintillation cocktail (Fisher Scinti Verse E, 10 mL). The results are shown in Table 1. The solvents tested were hexane, ethyl acetate, dichloromethane, chloroform, carbon tetrachloride, and chloroform-methanol(2:1). Of the solvents tested, ethyl acetate provided the most consistently favorable partition, i.e. 99% or better of the radioactivity was partitioned into the ethyl acetate. The extraction efficiency of ethyl acetate in a urine matrix is greater than that in a mixed feed matrix where a recovery of 87.0% was observed(1). In addition to its efficiency, ethyl acetate was the solvent of choice in subsequent recovery experiments because of its polarity. It is anticipated that at least some of the more polar metabolites of T-2 toxin will also be recovered.

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Table 1

Partition of ^{3}H-T-2 Toxin between Organic and Aqueous Phases[1]

		water	urine	sat'd NaCl soln.
hexane	(org)	23	10	94
	(aq)	77	90	6
ethyl acetate	(org)	99	99	99.6
	(aq)	1	1	0.4
dichloromethane	(org)	97	99	99.7
	(aq)	3	1	0.3
chloroform	(org)	92	95	98
	(aq)	8	5	2
carbon tetrachloride	(org)	10	11	35
	(aq)	90	89	65
chloroform-methanol	(org)	95	83	98
(2:1)	(aq)	5	17	2

[1] expressed as percentages of the total radioactivity

b. Effect of T-2 Toxin Quantity on Recovery

To test the effect of T-2 toxin concentration on the recovery efficiency of ethyl acetate, various quantities of T-2 toxin were spiked with ^{3}H-T-2 toxin and partitioned between urine and ethyl acetate. Stock solutions of T-2 toxin in methanol (1.0 mg/mL and 0.1 mg/mL) were prepared, and aliquots of these solutions ranging in size from 100 µL to 1 µL were spiked with 9.3 µL of ^{3}H-T-2 toxin in methanol (20 ng, 0.5 µCi in each aliquot). At the lower concentrations only the radiolabelled T-2 toxin was used. A calculated amount of methanol was added to each sample so that the total methanol concentration would be constant (ca. 100 µL). Each sample was partioned against ethyl acetate (1 mL) and urine (1 mL). The layers were separated, and 0.5 mL of each layer was counted in scintillation cocktail (Fisher Scinti Verse E). The results are shown in Table 2. Within the range of T-2 toxin levels from 1 ng to 10,000 ng, the recovery of radioactivity in ethyl acetate was 99%. The relatively constant extraction efficiency over a wide range of concentrations was as expected from the Law of Distributions. Thus, ethyl acetate would be an effective solvent for the extraction of nanogram and subnanogram quantities of T-2 toxin from urine.

Table 2

Effect of T-2 Toxin Quantity on Recovery

Labeled T-2 (ng)	Unlabeled T-2 (ng)	Total T-2 (ng)	ethyl acetate CPM	ethyl acetate %	Urine CPM	Urine %
20	9980	10000	208186	98.8	2561	1.2
20	2980	3000	214438	98.9	2397	1.1
20	980	1000	213189	99.0	2048	1.0
20	280	300	214477	99.0	2253	1.0
20	80	100	212493	99.0	2164	1.0
20	10	30	200843	98.8	2352	1.2
10	0	10	89545	98.8	1059	1.2
3	0	3	33910	98.6	491	1.4
1	0	1	11155	99.0	112	1.0

c. Stability of T-2 Toxin Solutions

A requirement in quantitative mass spectrometry is the use of an internal standard. A convenient form for the internal standard is a solution of known concentration, but it is also necessary that this solution have long term stability against decomposition. In order to test the long term stability of solutions of T-2 toxin against hydrolysis (the most likely route of decomposition in alcoholic and/or aqueous solutions), a sample of T-2 toxin in a methanol-buffer solution was followed by TLC with time.

Methanol (1 mL) containing ^{3}H-T-2-toxin (46 μCi) was added to 0.05M phosphate buffer (1 mL, pH = 7.00). The mixture was allowed to stand at ambient temperature. Aliquots (2 μL) were periodically removed and spotted on a TLC plate (Analtech Uniplate Silica gel GF). The plate was eluted with chloroform-methanol(9:1), and the silica bed was marked off in 17 horizontal strips of 0.5 cm each in width. Each strip was scraped off (1-2 drops of toluene were added on each strip to reduce losses from static electricity) and counted using scintillation cocktail (Fisher Scinti Verse E). The results are *summarized* in Table 3. *In* all cases the first fraction was considered background radiation and was subtracted from all other fractions in the series. The bulk of the radioactivity was present in fractions 15 and 16 which contain the T-2 toxin. With time the percentage of the total radioactivity in fractions 15 and 16 decreases while the percentage of radioactivity in fractions 11 and 12 increases. There is no significant variation in the other fractions. Fractions 11 and 12 correspond in R_f to HT-2 toxin. After 23 days nearly 73% of the total radioactivity remains in the fractions corresponding to T-2 toxin. The only significant hydrolysis product appears to be HT-2 toxin. If one assumes that the hydrolysis of T-2 toxin to HT-2 toxin is a pseudo-first order reaction under the present conditions, then a plot of the decrease of T-2 toxin with time can be used to estimate the half-life of T-2 in the medium. A plot of the log of the percentage of T-2 toxin versus time in days (Figure 6) reveals that in alcoholic-aqueous buffer solution at pH = 7.0, the half-life of T-2 toxin is estimated to be 50 to 60 days. Thus, T-2 toxin is quite stable, and storage of solutions of T-2 toxin in alcoholic solutions at low temperature in the absence of aqueous media is adequate.

Table 3
Distribution of radioactivity in the TLC analysis of ^{3}H-T-2 toxin in alcohol-buffer solution

		0 days		1.8 days		5.8 days		23 days	
fraction	Rf	CPM	%	CPM	%	CPM	%	CPM	%
1	.06	0	0.0	0	0.0	0	0.0	0	0.0
2	.11	37	0.1	89	0.2	69	0.2	63	0.1
3	.17	73	0.2	160	0.4	118	0.3	123	0.3
4	.23	234	0.5	224	0.5	346	0.9	227	0.6
5	.28	163	0.3	97	0.2	129	0.3	187	0.5
6	.34	79	0.2	97	0.2	38	0.1	87	0.2
7	.40	17	0.0	36	0.1	41	0.1	148	0.4
8	.45	19	0.0	108	0.3	87	0.2	110	0.3
9	.51	89	0.2	38	0.1	40	0.1	101	0.2
10	.57	37	0.1	59	0.1	133	0.3	688	1.7
11	.62	89	0.2	178	0.4	2347	6.1	7063	17.9
12	.68	350	0.8	1388	3.3	510	1.3	715	1.8
13	.74	353	0.8	203	0.5	139	0.4	208	0.5
14	.80	215	0.5	555	1.3	585	1.5	895	2.3
15	.85	529	1.1	28520	68.7	16490	42.7	7488	19.0
16	.91	43327	94.2	9774	23.5	17429	45.2	21233	53.9
17	1.0	390	0.8	12	0.0	75	0.2	85	0.2
Total		46001		41538		38576		39421	

d. Recovery of T-2 Toxin Hydrolysis Products

In addition to isolating T-2 toxin, a generalized recovery procedure should isolate metabolites of T-2 toxin from the urine matrix. Metabolic pathways of T-2 toxin have been studied using animal tissues(1). In addition to hydroxylation of the C-8 isovaleroxy ester of T-2 toxin(2), there is significant deacylation of T-2 to HT-2 toxin to 4-deacetylneosolaniol and ultimately to T-2 tetraol(3).

Interestingly, the chemical hydrolysis of T-2 toxin follows a similar pathway(3). The primary hydrolysis product of T-2 toxin is HT-2, and after longer reaction times the final product is T-2 tetraol. One can therefore test recovery procedures for metabolites of T-2 toxin by using the hydrolysis products. We have shown previously that recovery of ^{3}H-T-2 toxin from aqueous solutions and urine with ethyl acetate is 99% or better. To test if ethyl acetate is as efficient at recovering potential T-2 toxin metabolites, we followed the hydrolysis of ^{3}H-T-2 toxin with methanolic ammonia and partitioned aliquots of the hydrolysate against ethyl acetate and water.

A methanol solution (0.5 mL) of ^{3}H-T-2 toxin (23 μCi) was added to 1M NH_4OH in methanol-water (4:1) (0.5 mL). At various time intervals a 10 μL aliquot of the solution was added to a mixture of ethyl acetate (1 mL) and water (1 mL). The mixture was shaken and the layers were separated. A portion (0.5 mL) of each layer was counted for 2 min in scintillation cocktail (Fisher Scinti Verse E). The results are shown in Table 4. The data indicate that there is a time-dependent decrease in the total amount of radioactivity that is partitioned into the ethyl acetate. During the first six hours of reaction the efficiency of ethyl acetate in extracting the total radioactivity decreases by less than 3%. However, with longer reaction times the efficiency of extraction decreases markedly. (See Figure 7).

If ethyl acetate had been as efficient in extracting the hydrolysis products as it was in extracting T-2 toxin, then the total amount of radioactivity in the ethyl acetate layer would have varied little. The

hydrolysis products which accumulate with time have a less favorable partition coefficient than the parent compound, resulting in the observed time-dependent decrease in the extraction with ethyl acetate. Since the final hydrolysis product is T-2 tetraol, this decrease is expected to level off to a constant value. To compensate for this loss of efficiency, extraction with multiple portions of ethyl acetate and salting out with sodium chloride is recommended.

The extraction efficiency of ethyl acetate for the hydrolysis products was compared in different matrices at a specific point during the hydrolysis reaction. When an aliquot of the hydrolysis reaction mixture was partitioned between distilled water and ethyl acetate, 68.7% of the total radioactivity was located in the organic layer. The amount of radioactivity in the organic layer increased to 71.2% of the total when urine, which contains naturally occurring salts, was used instead of distilled water. The use of saturated sodium chloride solution increased the radioactivity in ethyl acetate to 81.4% of the total.

Since the efficiency of ethyl acetate is low for the recovery of the polar hydrolysis products, the partition was attempted with n-butanol. n-Butanol has the advantage of being much more polar than ethyl acetate and unlike methanol it is immiscible with water. Whereas after 288 hours of reaction the ethyl acetate was able to recover only 10% of the total radioactivity, n-butanol was able to recover nearly 48%. If saturated sodium chloride is used, the recovery is 78%. Thus extraction of aqueous solutions saturated with sodium chloride with n-butanol is expected to recover T-2 toxin as well as its hydrolysis/metabolic products in very good yield. (See Figure 7 and Table 4.)

References

1. Y. Ueno, Pure & Appl Chem, 58, 339 (1986).

2. T. Yoshizawa, T. Sakamoto and K. Okamoto, Appl. Environ. Microbiol., 47, 130 (1984).

3. T. Yoshizawa and N. Morooka, Appl. Microbiol., 30, 38 (1975).

Table 4

Partition of T-2 toxin hydrolysis products

	ethyl acetate		water	
time (hr)	CPM	% of Total	CPM	% of Total
0	111468	98.0	2320	2.0
0.5	113913	97.5	2886	2.5
1.0	110812	97.0	3388	3.0
1.5	121710	97.2	3527	2.8
2.0	118669	96.7	4099	3.3
2.5	119725	96.9	3886	3.1
3.0	117865	96.6	4135	3.4
3.5	115513	96.2	4605	3.8
4.0	114552	96.3	4381	3.7
6.0	112480	95.4	5362	4.6
24.0	99314	86.8	15126	13.2
48.0	81427	72.2	31346	27.8
120.0	38544	36.9	65817	63.1
172.0	22650	22.4	78612	77.6
288.0	9949	9.9	90728	90.1
	n-butanol		water	
172.0	57784	53.5	50322	46.5
288.0	52708	47.6	58033	52.4
	n-butanol		sat'd NaCl	
288.0	81883	77.5	23816	22.5

3. CHROMATOGRAPHIC SEPARATIONS

a. Pre-Column Cleanup Techniques

The information obtained from the extraction techniques for the isolation of mycotoxins from urine discussed in the previous section is fundamental to the analysis of those analytes by HPLC/MS. In order to further improve the efficiency of the analytical process we thought it would be worthwhile to explore the feasibility of pre-column cleanup procedures using in part the information derived from these extraction techniques.

The basic idea behind the pre-column cleanup system designed for these analyses, is to provide an on-line isolation process for the mycotoxins and thereby minimize the need for prior elaborate extractions or manual handling of these potentially toxic materials. A similar technique was devised previously in our laboratories and was applied successfully to the analysis of drugs from serum (A. Nazareth, L. Jaramillo, B.L. Karger, and R.W. Giese, J. Chromatogr., 309, 357 (1984). This technique would provide a foundation for a more efficient development of a pre-column system that would encompass the compounds of diverse polarity ranging from T-2 toxin to T-2 tetraol.

A schematic of the pre-column cleanup system is shown in Figure 8. Effectively, the sample loop has been replaced by an extraction column connected to a six-port injection valve. A second precolumn has been placed before the analytical column for its protection. The typical operation of the system involves the following four steps:

(i) preconditioning of the extraction column by injection of 2.5 mL 10% acetonitrile in water.

(ii) injection of urine sample containing trichothecenes; the analytes along with other impurities are trapped on the extraction column.

(iii) removal of the bulk of the impurities to waste by washing with 2.5 mL 10% acetonitrile

(iv) *switching the extraction column into the system*

(v) elution with 50% acetonitrile/water and introduction into the analytical column.

All of these operations are conducted by appropriate switching of the six-port injection valve.

The trichothecenes T-2 and HT-2 were selected as test analytes to determine the efficiency of the system. The chromatographic conditions were as follows:

System:	Waters solvent delivery system M 6000A Kratos Spectroflow 773 variable UV-detector, operated at 200 nm, 0.05 AUFS Rheodyne Injector 7125, (10 microliter sample loop replaced by pre-column or pre-column) recorder: linear instruments 255/MM integrator: HP 3358 A automation system	
Solvents:	elution: 50% acetonitrile/water wash: 10% acetonitrile/water	
Columns:	precolumns:	20 x 3.9 mm filled with Bondapak C-18 Corasil (Waters)
	main column:	Partisil ODS-3 5 micrometer (Whatman) $t_o = 1.95$ min

As noted previously, a UV detector was used throughout and, the poor UV characteristics of the analytes forced us to operate at the 200 nm wavelength where background interferences are plentiful. Nevertheless, this still permits a reasonable assessment of the operating characteristics of the system.

Figure 9 shows the chromatogram of a sample of pure T-2 (360 ng) by direct injection into the analytical HPLC column, i.e., by-passing the extraction pre-column. This can be compared to the analysis of an identical amount of T-2 (Fig. 10) which was first collected in the extraction column, washed with 10% acetonitrile and finally, introduced into the analytical column and eluted using 50% acetonitrile. Comparison of the two signals indicates more than 95% recovery of the analyte. The same experiment with pure HT-2 also shows over 95% sample recovery. Compare, for example, the signals in the chromatograms of Figure 11 (300 ng HT-2 analyzed without precolumn extraction) and Figure 12 (300 ng HT-2 analyzed following precolumn extraction).

Analysis of T-2 and HT-2 in urine was tested next using the same procedures. A blank of 10 μL urine was first injected into the extraction precolumn and then eluted without a prewash, into the analytical column (Figure 13). As is evident from the chromatogram of Figure 13, the urine impurities would prohibit the detection of T-2 or HT-2. On the other hand, when the transfer to the analytical column is preceded by a prewash of the extraction column with 2.5 mL 10% acetonitrile, a better baseline can be obtained for blank urine as shown in Figure 14. Indeed, detection of T-2 and HT-2 in urine was quite feasible with recoveries of about 90% for T-2 and nearly 100% for the case of HT-2 as shown in Figures 15 and 16, respectively. Standard deviations of 11% for T-2 and 5% for HT-2 were observed in these analyses. The results of these experiments suggest that, even with the use of a low UV detection, it is realistic to project detection limits of ~30 ng for the two analytes using these simple precolumn techniques. Based on results in section 2, use of butanol could provide a more general system for the concurrent recovery of T-2 and its more polar byproducts. If preceded with an efficient conventional extraction, we should be able to remove most of the urine matrix impurities and further reduce the background noise. Furthermore, given the high sensitivity and selectivity of the mass spectrometric detector, it is realistic to anticipate orders of magnitude improvement in detection limits as we proceed with the HPLC/MS studies. We are now in the process of starting this phase of the work.

b. Chromatographic Separation of T-2 and Potential Metabolites

The expected metabolic products from T-2 are likely to be produced by de-acetylation processes which should yield hydroxylated compounds covering a wide range of polarities. As a result, gradient reversed phase HPLC is required in order to achieve their analysis within a reasonable span of time. In order to assess the optimal separation conditions, we determined systematically the retention times and k' values of the compounds in the series as a function of the concentration of organic modifier in water. As separations with k' values greater than 10 are of no practical meaning, only the region between k' of 0.5 and 10.0 was checked. The results for the tricothecenes T-2 tetraol, Nivalenol, DON, Verrucarol, HT-2, DAS and T-2 are tabulated in Table 5 and graphically displayed in Figure 17.

$$k' = \frac{t_r - t_o}{t_o}$$

where: t_o = retention time of inert peak
t_r = retention time of analyte

The presentation of Figure 17 shows two groups of distinct polarity characteristics within the series of T-2 and possible metabolites. The low polarity group HT-2, DAS, T-2 (dotted lines on Figure 17), shows reasonable retention times on chromatographic systems with 35-45% acetonitrile. Further optimization with mixtures of methanol/acetonitrile/water should result in an isocratic chromatographic system for the qualitative and quantitative determination of these substances. Thus, it should be possible to maximize the removal of interfering substances in the precolumn clean-up step described above by raising the content of the organic modifier acetonitrile up to 20% without any influence on the amount of recovered toxins.

With the exception of T-2 tetraol/Nivalenol we are able to achieve a good separation for the high-polarity group T-2 tetraol, Nivalenol, DON, Verracarol, (solid lines on graph of Figure 17). Again, an optimization of the eluents in a polarity range represented by 5 to 15% acetonitrile should result in an isocratic system for the separation of these substances. It is worth investigating the percentage of toxin-recovery by the precolumn clean-

up step using butanol. According to the k'-values, at 5% acetonitrile there is a good possibility that over 50% of the polar substances could be trapped on the sample-loop precolumn.

In summary, the data on Figure 17, show that a complete separation of all the indicated trichothecenes can only be achieved by gradient elution, probably in the range between 5% and 50% acetonitrile in water. These conditions will be employed in the forthcoming on-line HPLC/MS studies.

Table 5
Retention Times of Selected Trichothecenes

toxin	conc. (ng/µL)	(1) RT	k'	(2) RT	k'	(3) RT	k'
T-2 tetraol	58	10.1	4.3	5.3	1.8	4.0*	1.1*
Nivalenol	100	9.6	4.1	5.2	1.8	3.1*	0.6*
DON	100	20.5	9.8	9.0	3.7	4.7	1.5
Verrucarol	80			20.8	9.9	7.0	2.7

toxin	conc. (ng/µL)	(4) RT	k'	(5) RT	k'	(6) RT	k'
T-2 tetraol	58	3.0*	0.6*	2.7	0.4	2.5	0.3
Nivalenol	100	2.9*	0.5	2.7	0.4	2.6	0.4
				22.5		11.6	
DON	100	3.9	1.1	3.4	0.8	3.2	0.7
Verrucarol	80	4.5	1.4	3.7	0.9	3.4	0.8
				22.6		11.7	
HT-2	100	19.4	9.2	7.7	3.1	4.9	1.6
DAS	100	14.3	6.5	7.3	2.8	5.2	1.7
T-2	100	>30		18.0	8.5	8.7	3.6

toxin	conc. (ng/µL)	(7) RT	k'	(8) RT	k'
T-2 tetraol	58	2.8	0.5	2.7	0.4
Nivalenol	100	2.8	0.5	2.7	0.4
		5.5		8.5	
DON	100	3.1	0.6	2.9	0.5
Verrucarol	80	3.3	0.7	3.2	0.7
		5.5		8.6	
HT-2	100	3.4	0.8	3.8	1.0
DAS	100	3.6	0.9	4.2	1.2
T-2	100	4.4	1.3	5.6	1.9

columns: Precolumn: Waters Corasil C-18 (20*3.9 mm)
Analytical Column: Whatman Partisil ODS 3 (5 µm) (250*4.6 mm)

eluent:
(1) 5% acetonitrile/water
(2) 10% acetonitrile/water
(3) 20% acetonitrile/water
(4) 30% acetonitrile/water
(5) 40% acetonitrile/water
(6) 50% acetonitrile/water
(7) 50% acetonitrile/water
(8) 70% acetonitrile/water

flow: 1mL/min
UV: 205 nm, 0.05 AUFS
pressure: about 2500 psi
t0: 1.9 min
injection: 10µL each
*Indicates interference from system peaks

4. MASS SPECTROMETRY

We have conducted a preliminary survey of the mass spectrometric behavior of the target compounds. This work was carried out with the Finnigan 4000 mass spectrometer and the compounds were introduced into the MS *via* the moving belt interface. While the on-line HPLC/MS work will be done with our recently acquired VG-7070SE mass spectrometer, we felt that it was important to assess the general behavior of these molecules when they are vaporized off the polyimide surface of the belt. In particular we were interested in the occurrence of any catalytic effects from the belt on the spectral characteristics of the trichothecenes, since the VG-7070SE MS also employs the same interface for HPLC/MS.

For purposes of quantitative analysis, the best spectra were obtained with ammonia as the reagent gas. The NH_3-CI spectra are, for the most part, dominated by abundant molecular adduct ions, $[M+NH_4]^+$, which can be used during selective ion monitoring. (Table 6). In the Finnigan 4000 LC/MS system a flash vaporizer temperature of 350°C was required to introduce the compounds into the ion source. This appears to be an optimum temperature and a good compromise between the volatility requirements, the thermal tolerance of the belt material and the apparent thermal stability of the compounds themselves.

Table 6
Partial mass spectra ($CI-NH_3$) of T-2 and potential matabolites

T-2	T-2 Tetraol	HT-2	DON	DAS
484[a,b](100)[c]	316[a](20)	442[a](100)	314[a](64)	384[a](100)
182(30)	298(30)	425(18)	297(28)	324(20)
142(22)	250(20)	342(20)	249(100)	307(10)
122(45)	233(30)	215(20)	201(50)	
	215(100)		126(40)	

[a] $[M+NH_4]^+$ ion

[b] Numbers refer to m/z value

[c] Values in parentheses refer to % relative intensity

The sensitivity of the Finnigan LC/MS system was tested using T-2 toxin as a test analyte and selective monitoring of the m/z 484, molecular adduct ion. A linear response was observed in the range from 1μg to 20 ng and a good signal (5350 A/D) was obtained for as low as 12 ng of sample. While the detection of 1-5 ng of sample was possible, quantitative reproducibility at those levels was poor. It is likely that these problems are largely related to the configuration of the belt which passes outside the ion source volume. We expect that the VG configuration, in which the belt crosses the ion source volume, will resolve this problem and result in considerable improvement of the detection limits. Work along these lines is now in progress using the recently acquired VG7070SE system.

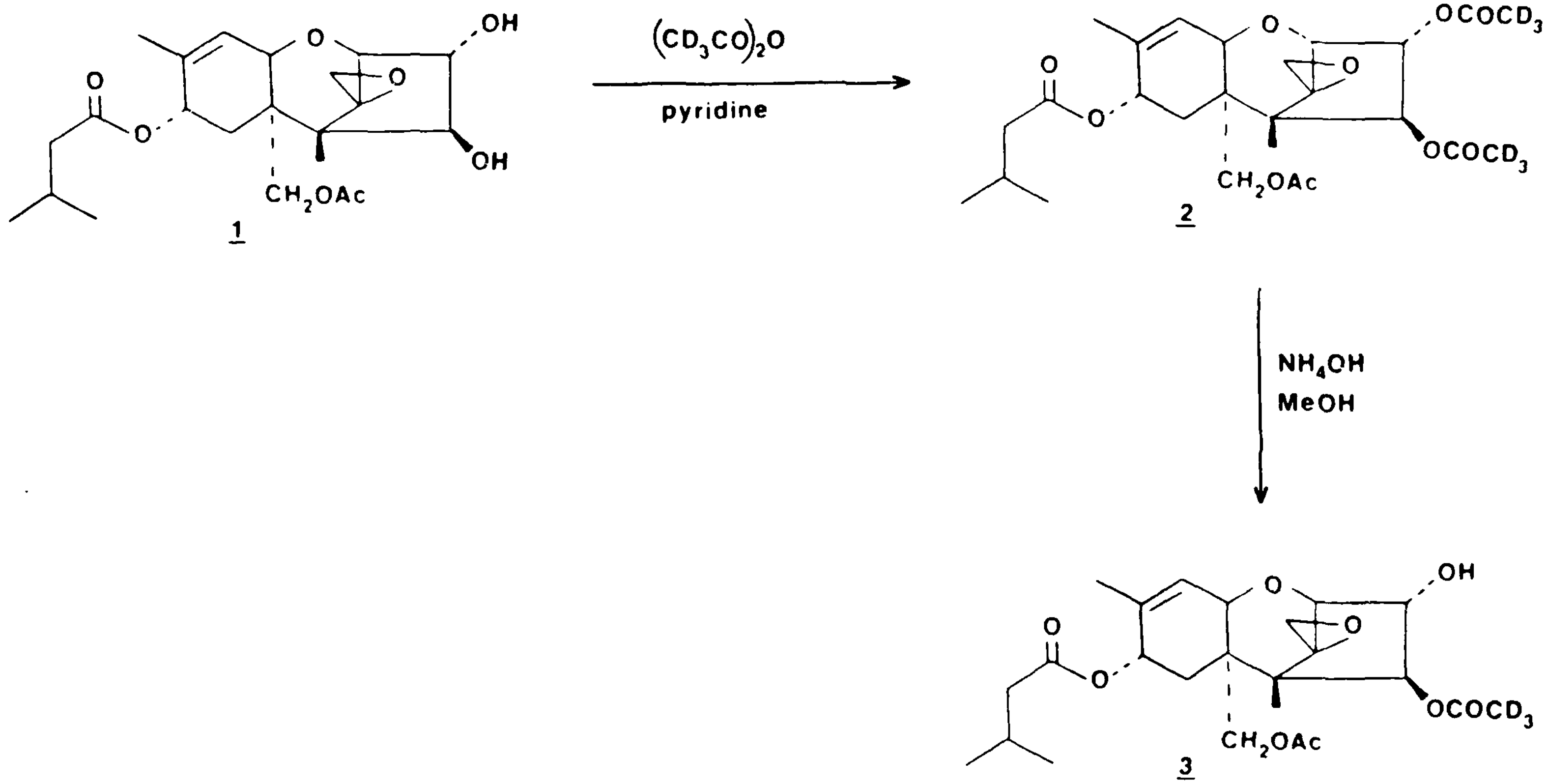

Figure 1 - Synthesis of d_3-T-2 toxin.

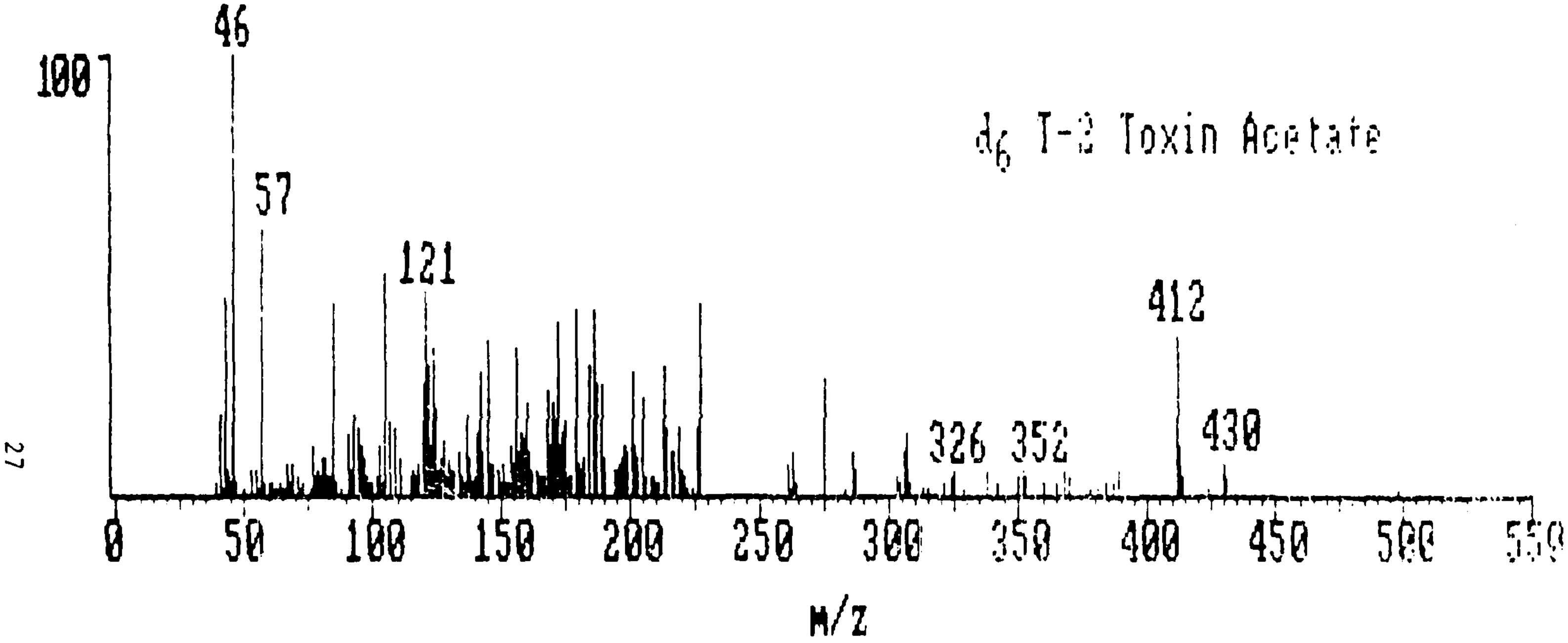

Figure 2 - Mass spectrum of d_6-T-2 toxin acetate.

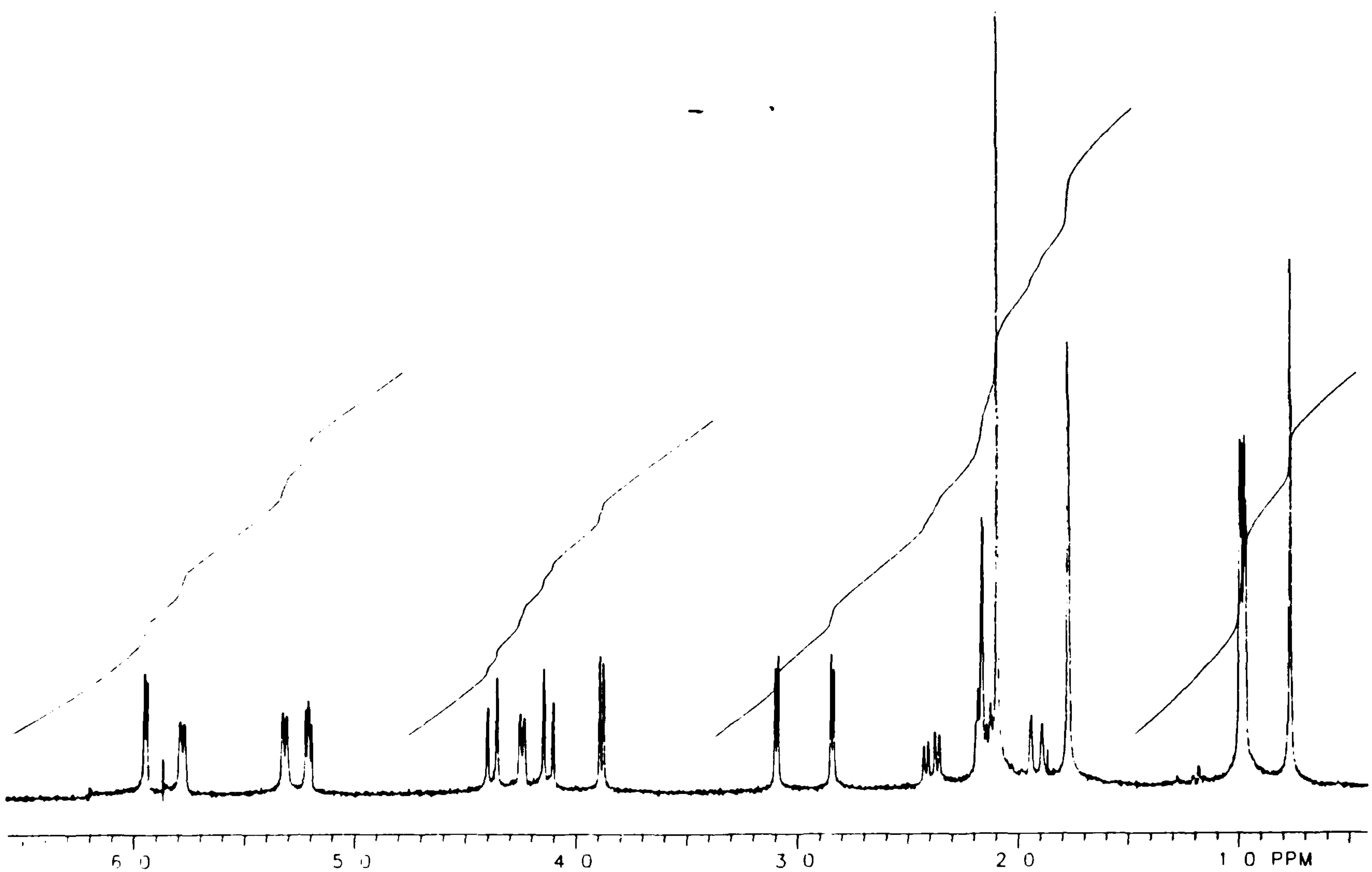

Figure 3 - NMR spectrum of d_6-T-2 toxin acetate.

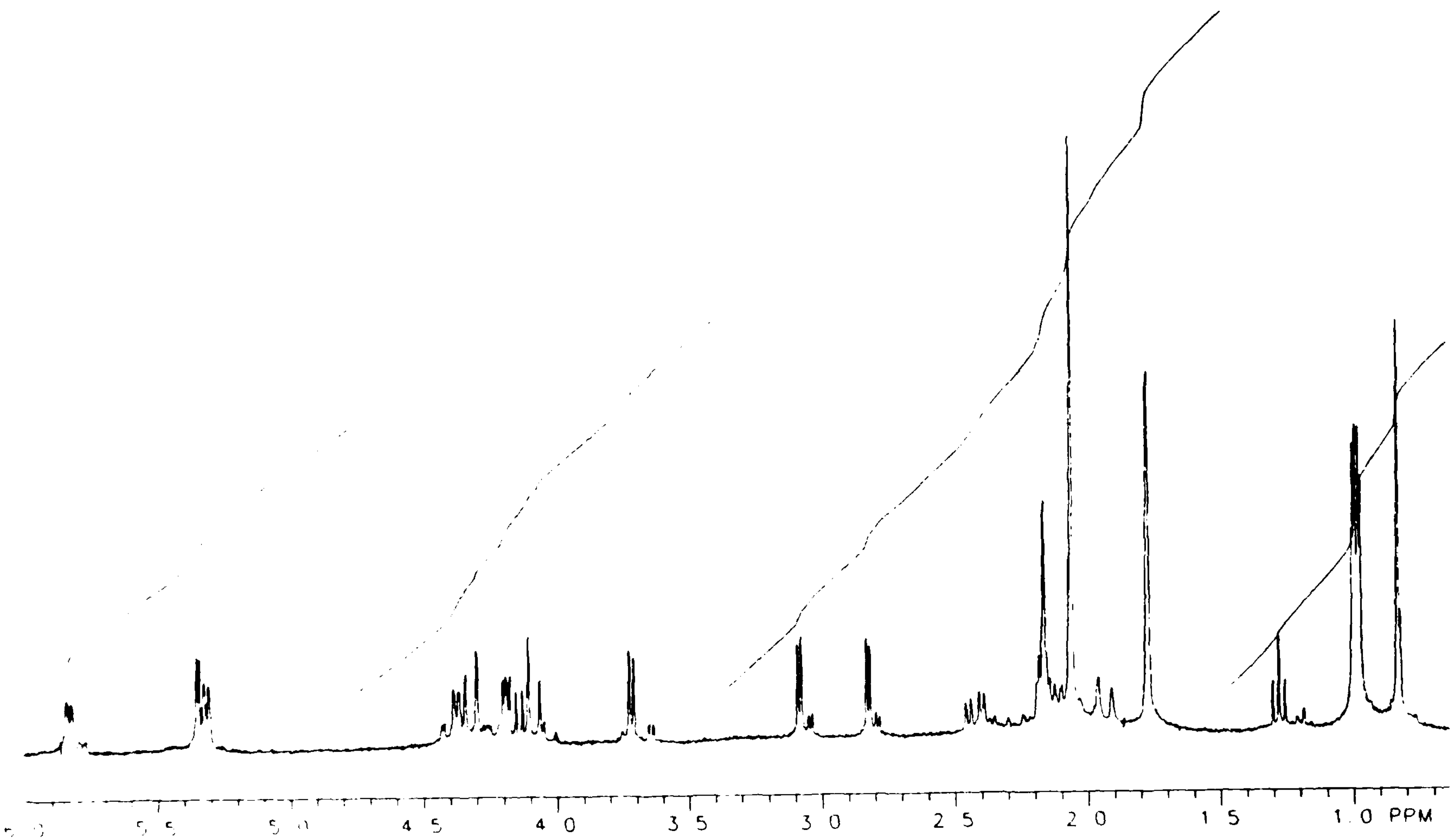

Figure 4 - NMR spectrum of d_6-T-2 toxin acetate hydrolysis reaction mixture.

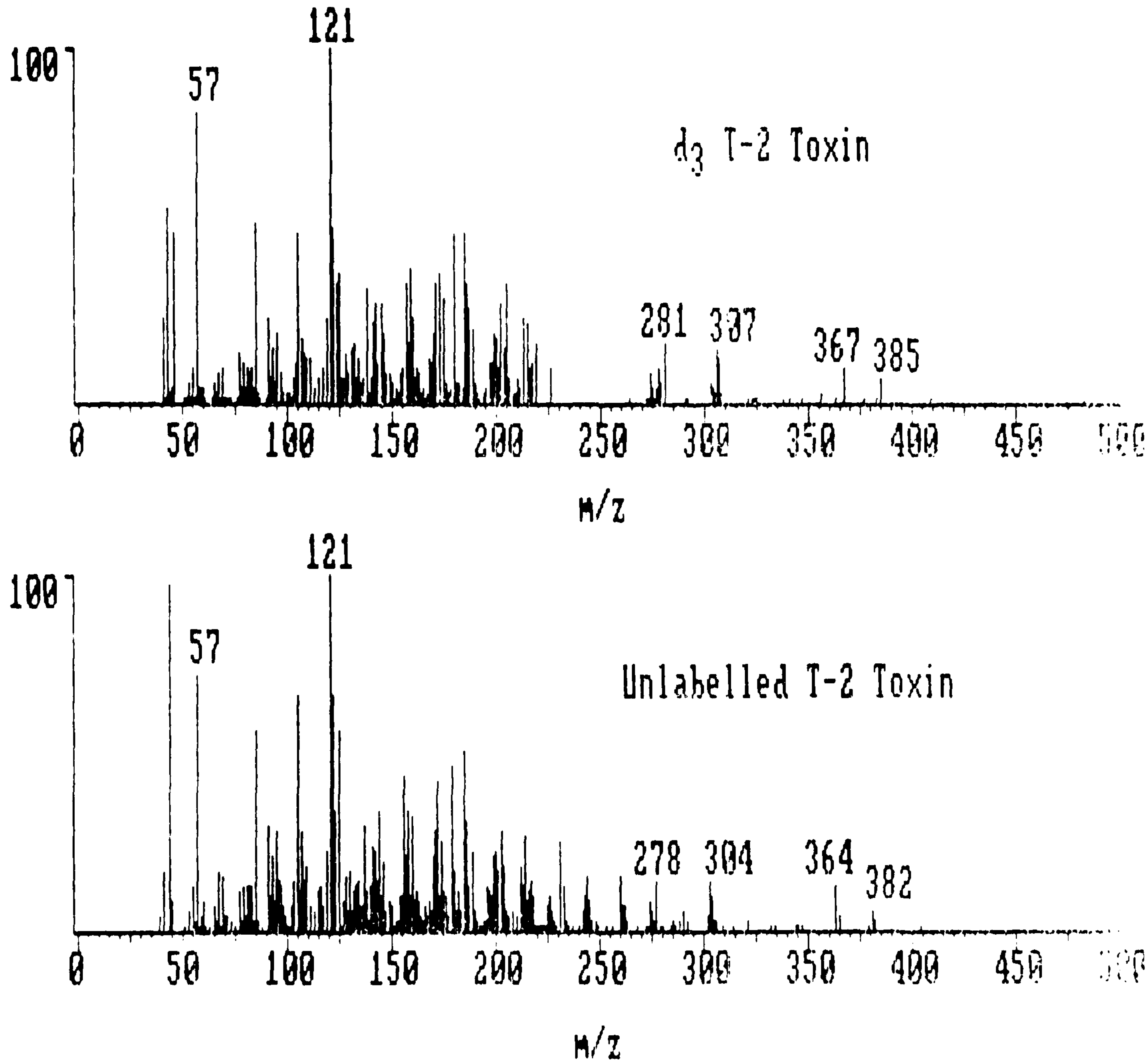

Figure 5 - Mass spectra of labelled and unlabelled T-2 toxin.

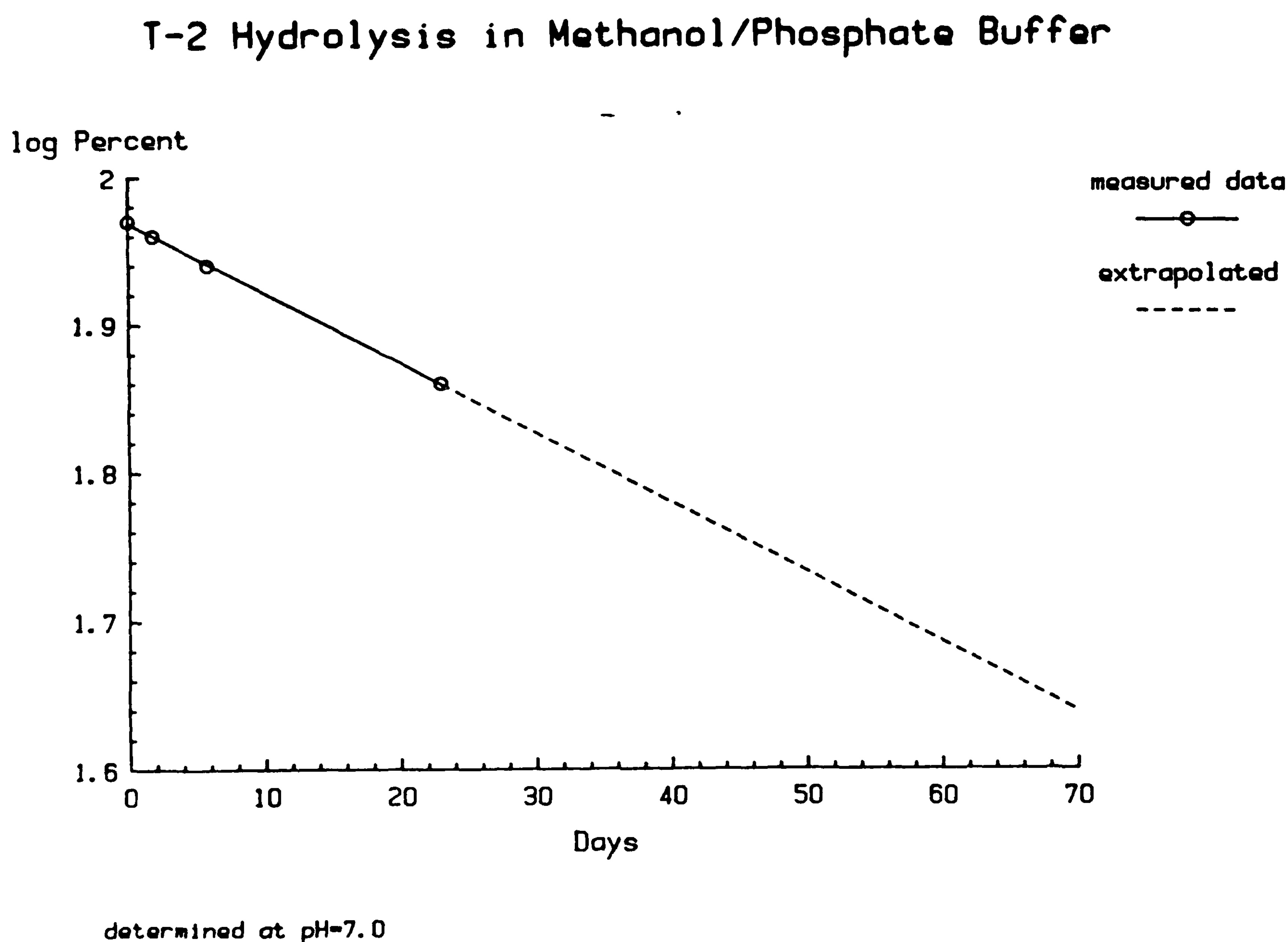

Figure 6 - T-2 Hydrolysis in Methanol/Phosphate Buffer

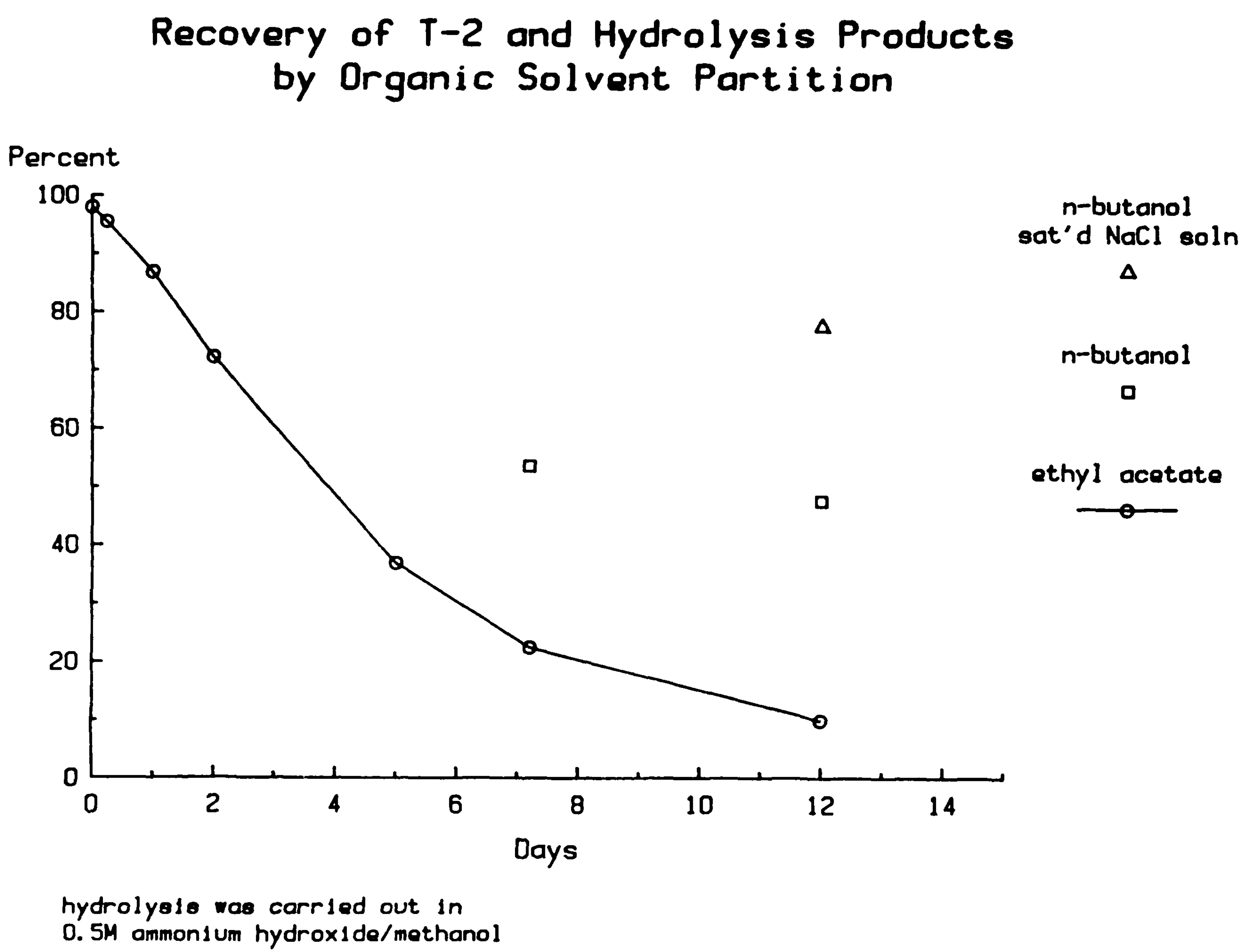

Figure 7 - Recovery of T-2 and Hydrolysis Products by Organic Solvent Partition

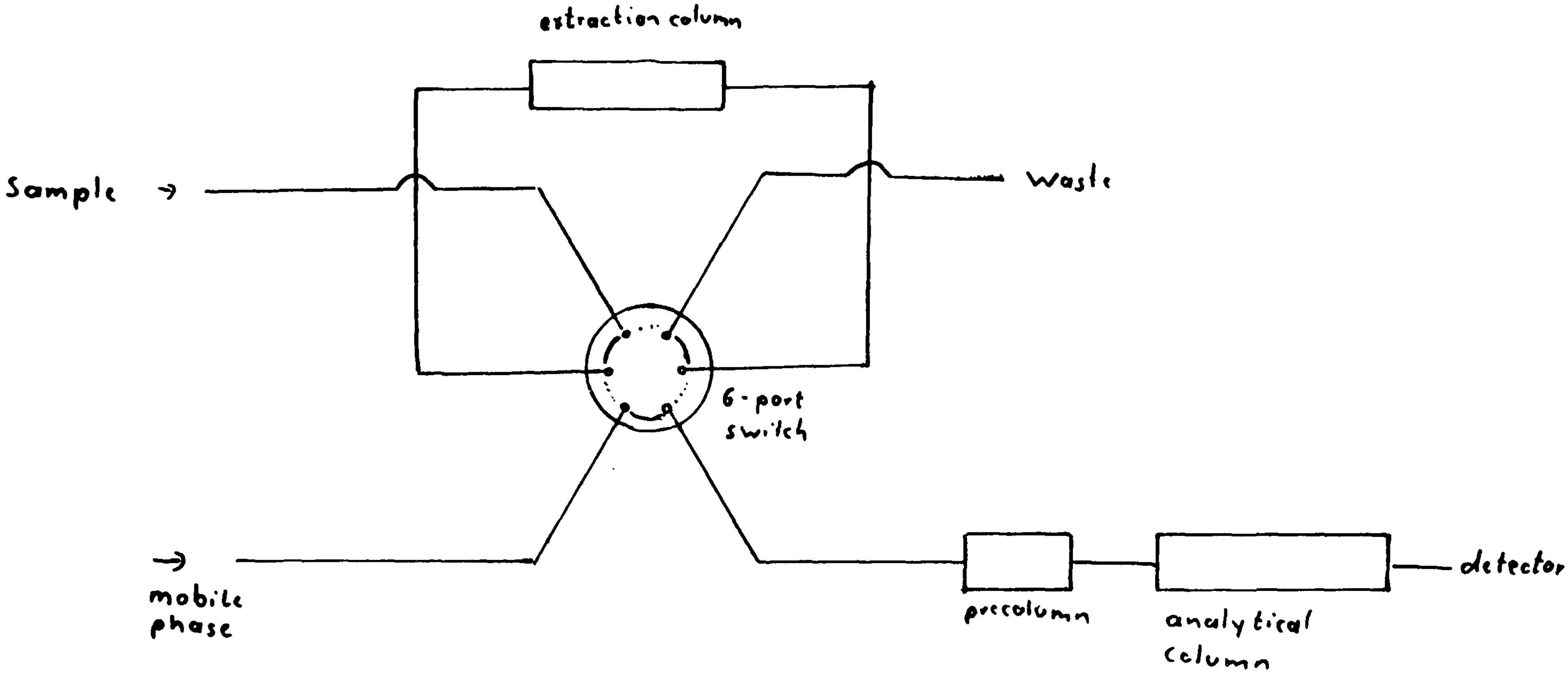

Figure 8 - Pre-column cleanup system.

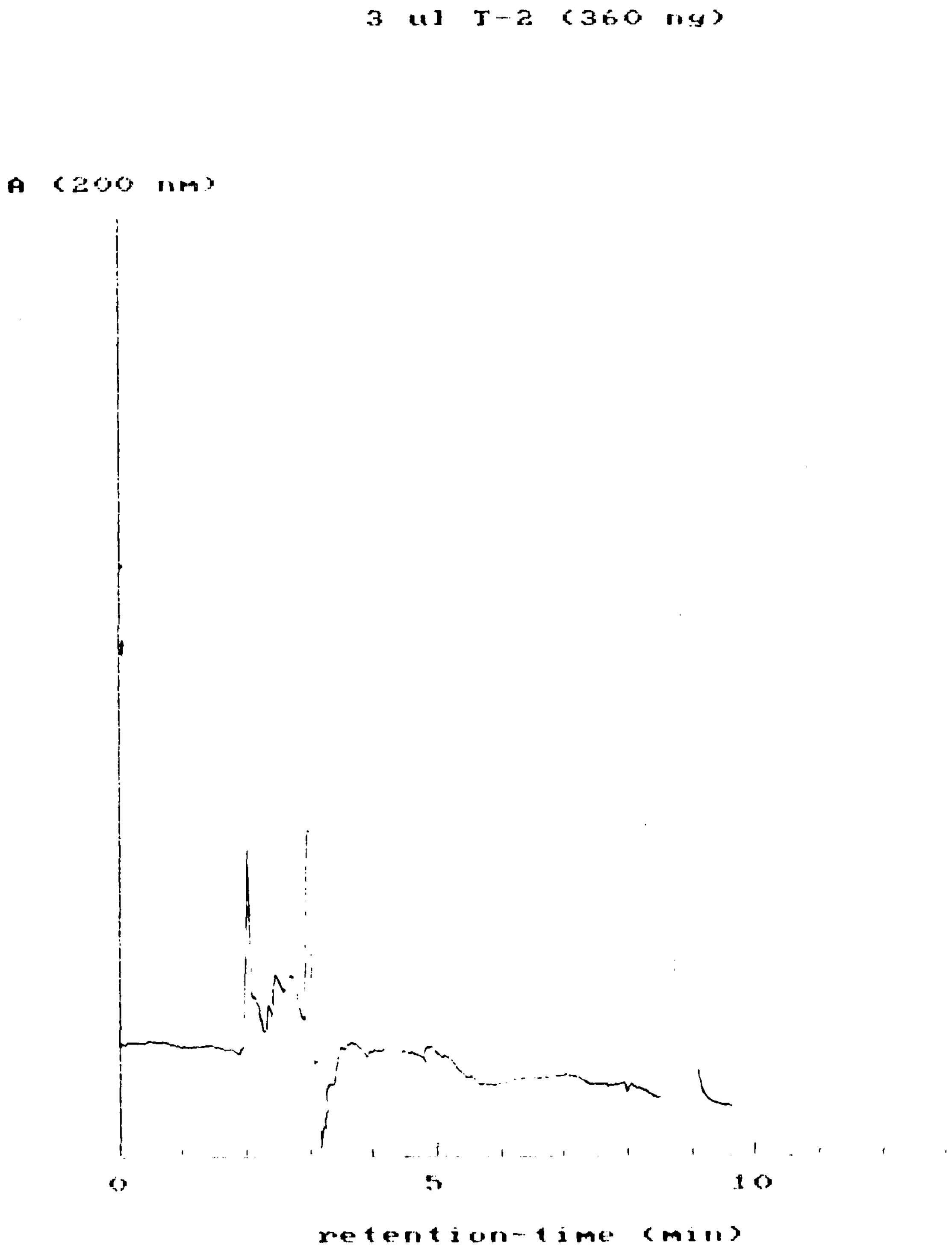

Figure 9 - HPLC chromatogram of T-2 toxin by direct injection.

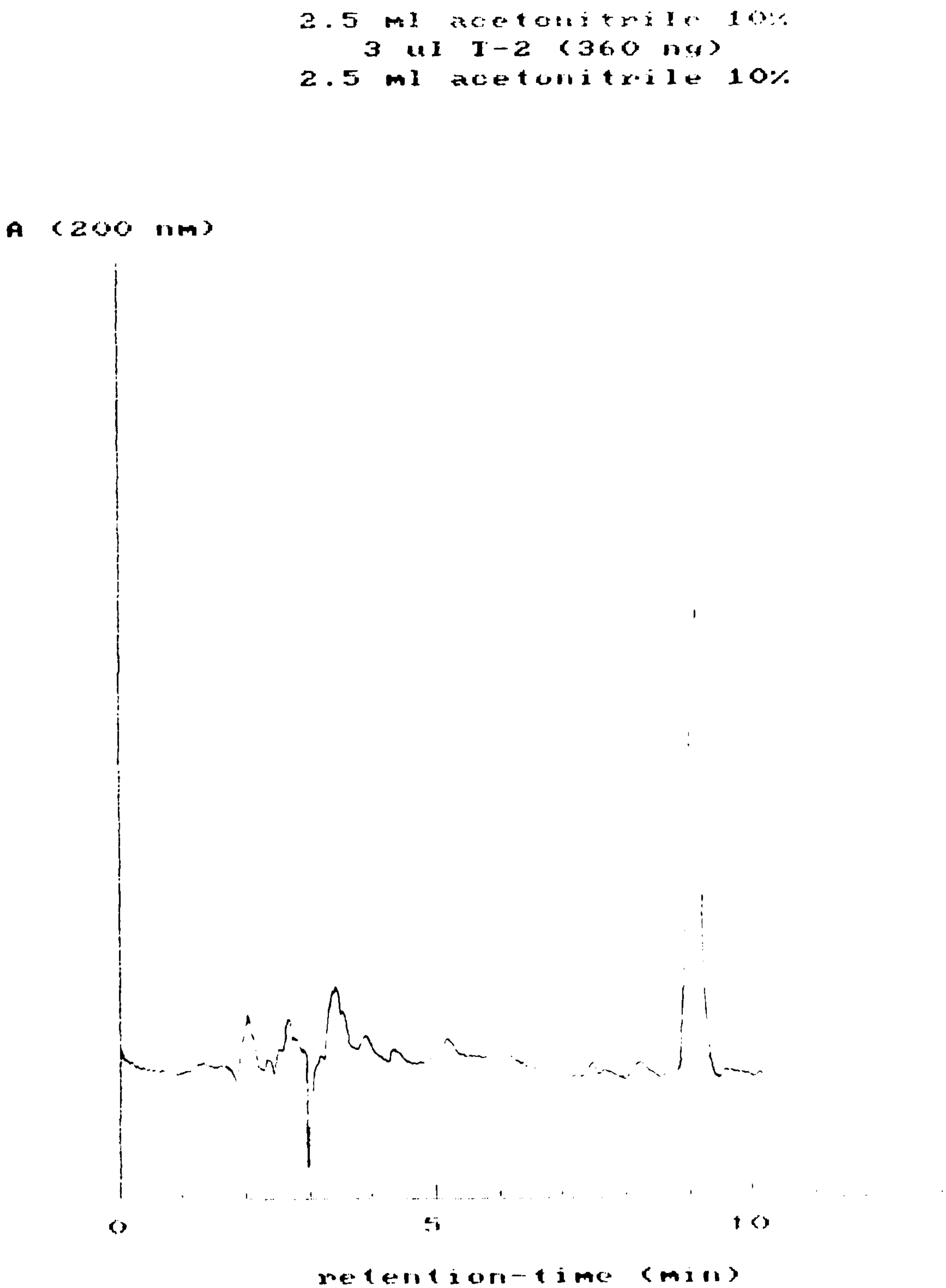

Figure 10 - HPLC chromatogram of T-2 toxin after pre-column extraction.

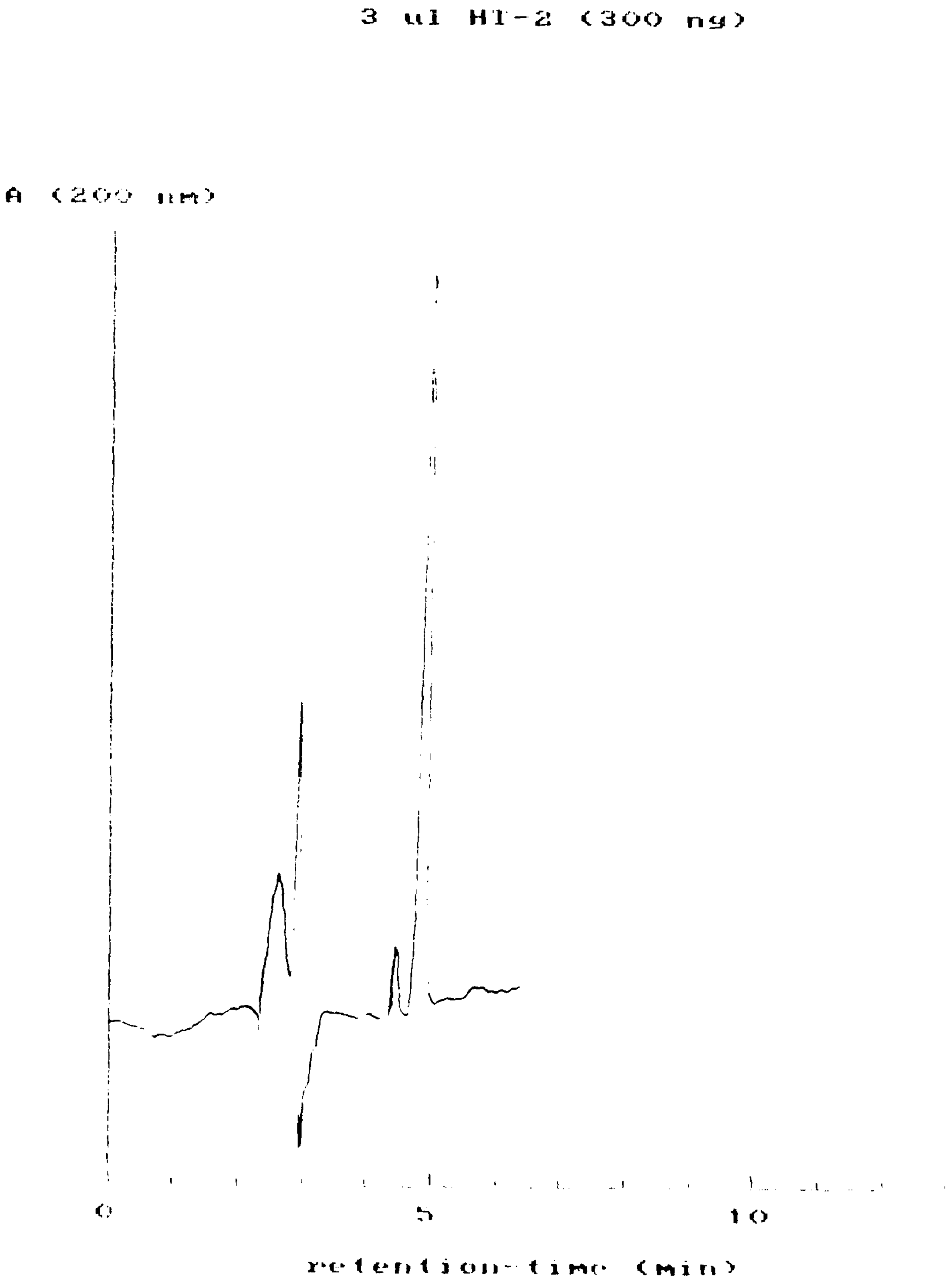

Figure 11 - HPLC chromatogram of HT-2 toxin by direct injection.

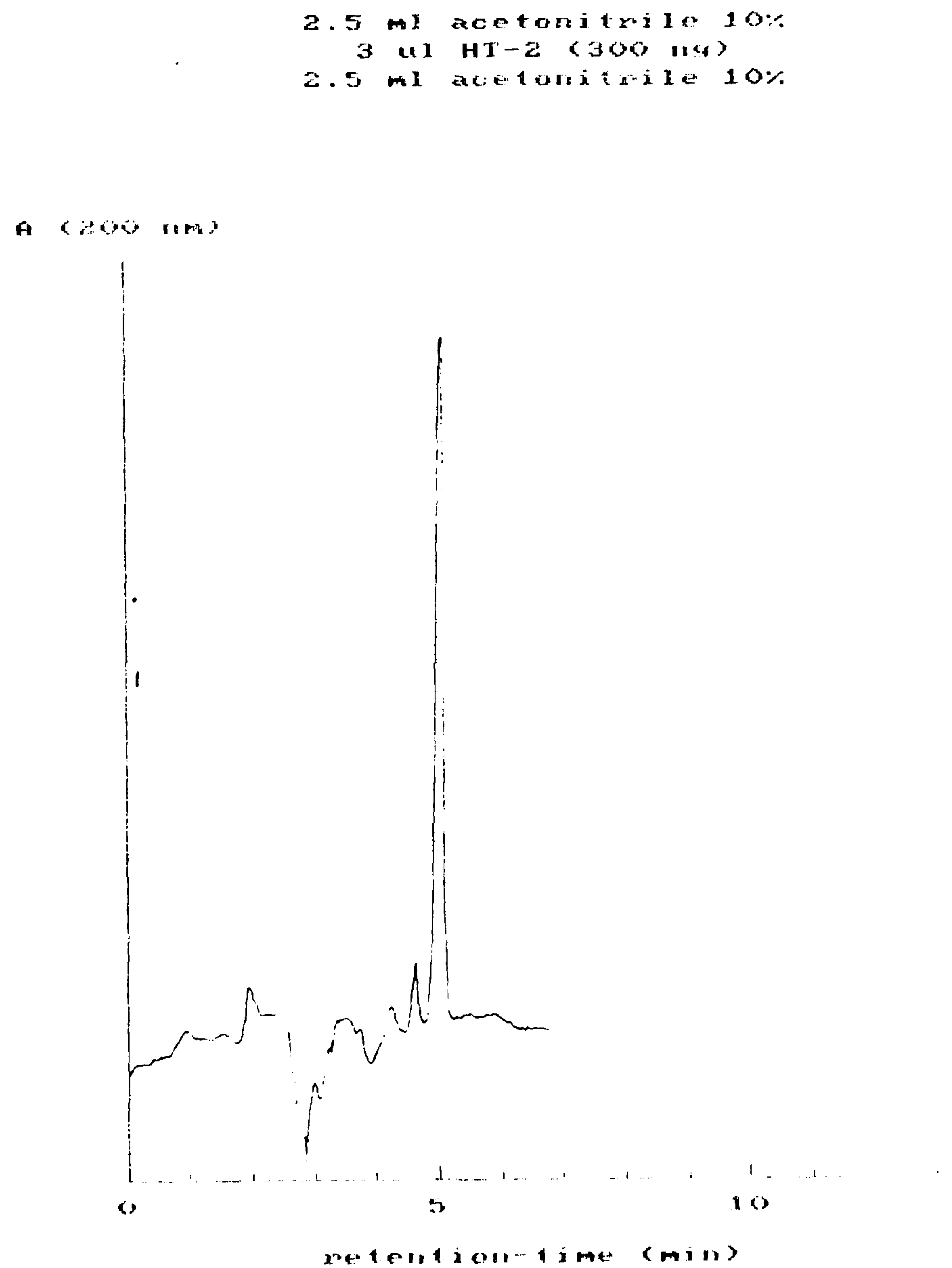

Figure 12 - HPLC chromatogram of HT-2 toxin after precolumn extraction.

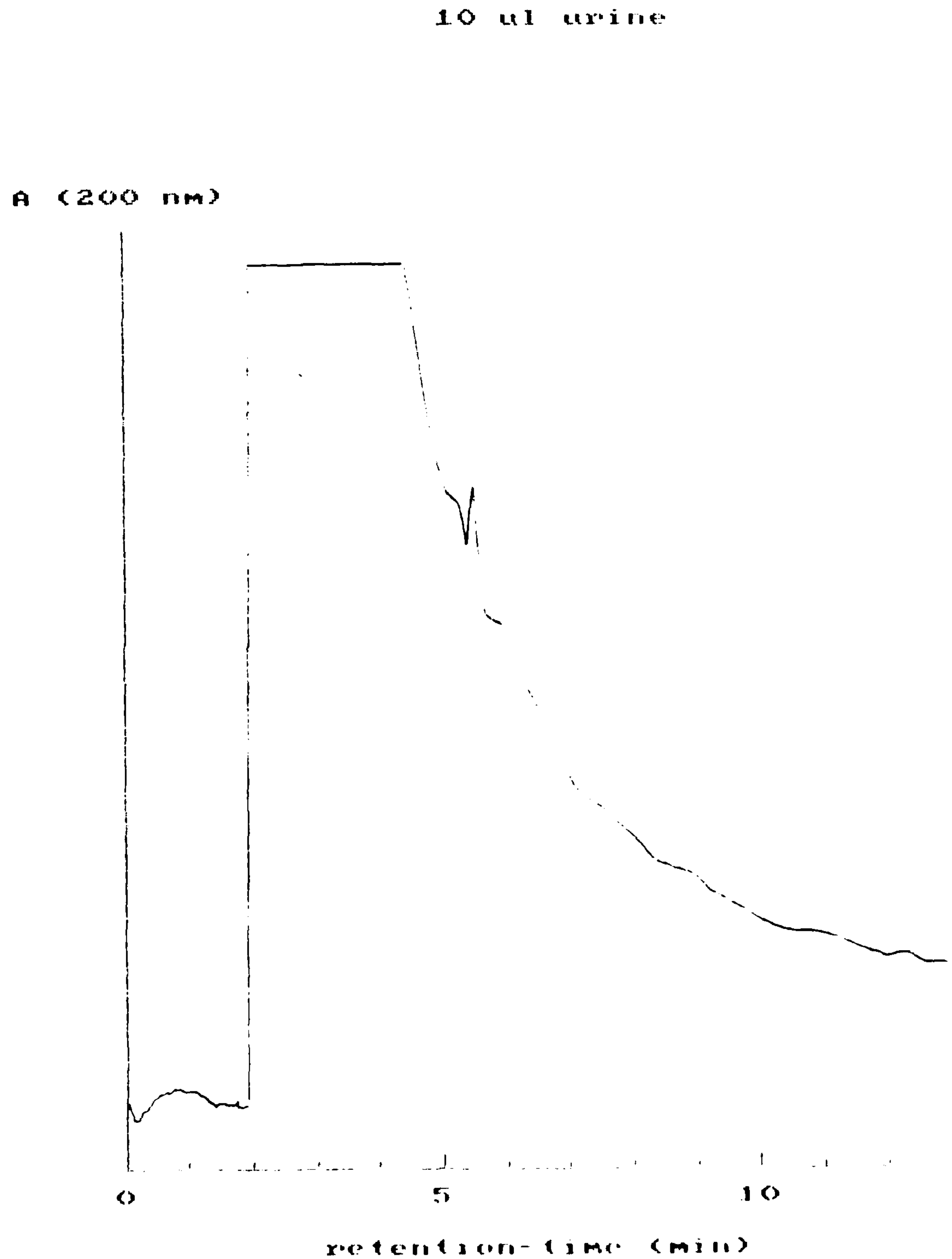

Figure 13 - HPLC chromatogram of urine blank without pre-column wash.

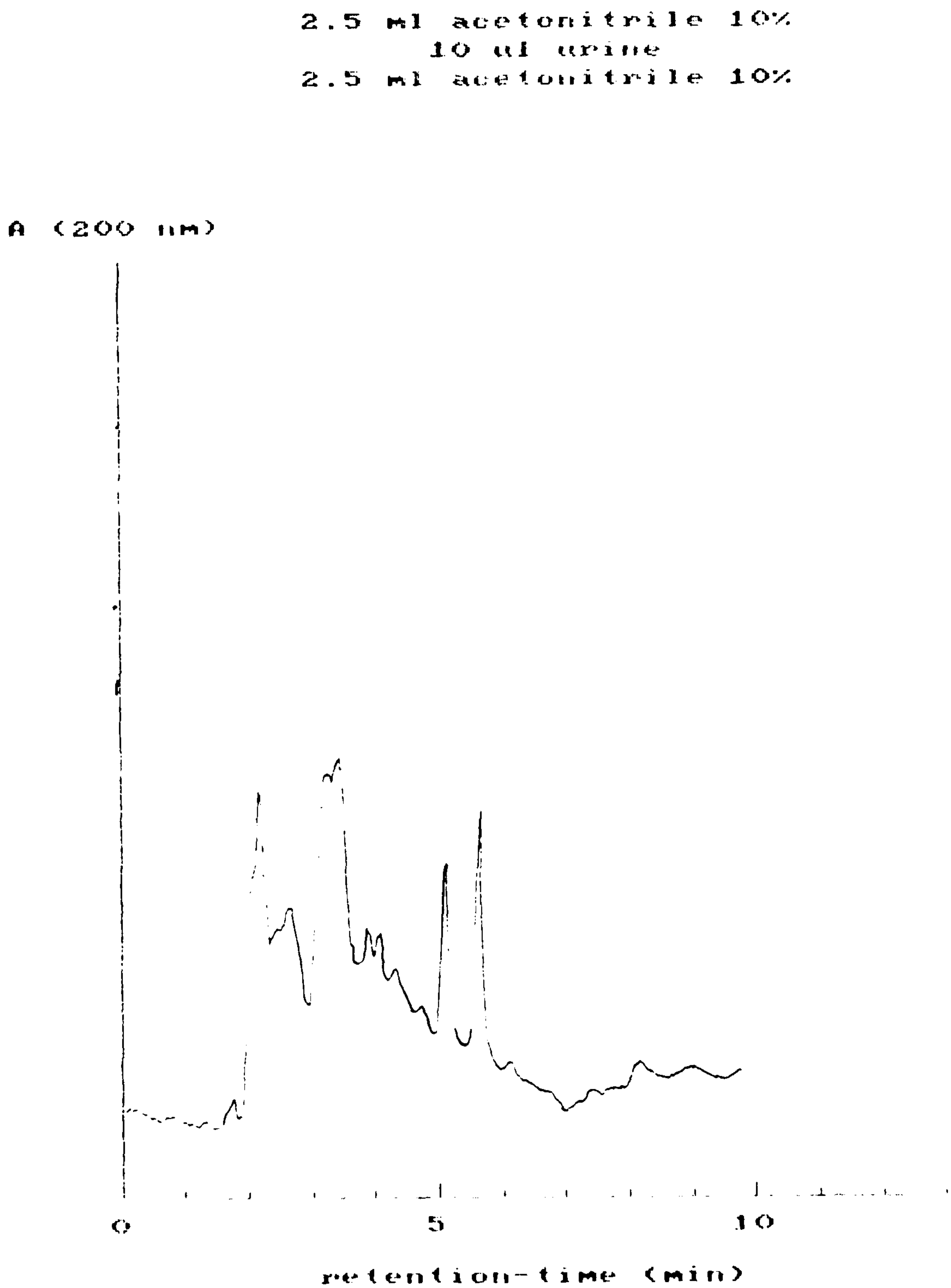

Figure 14 - HPLC chromatogram of urine blank after pre-column extraction.

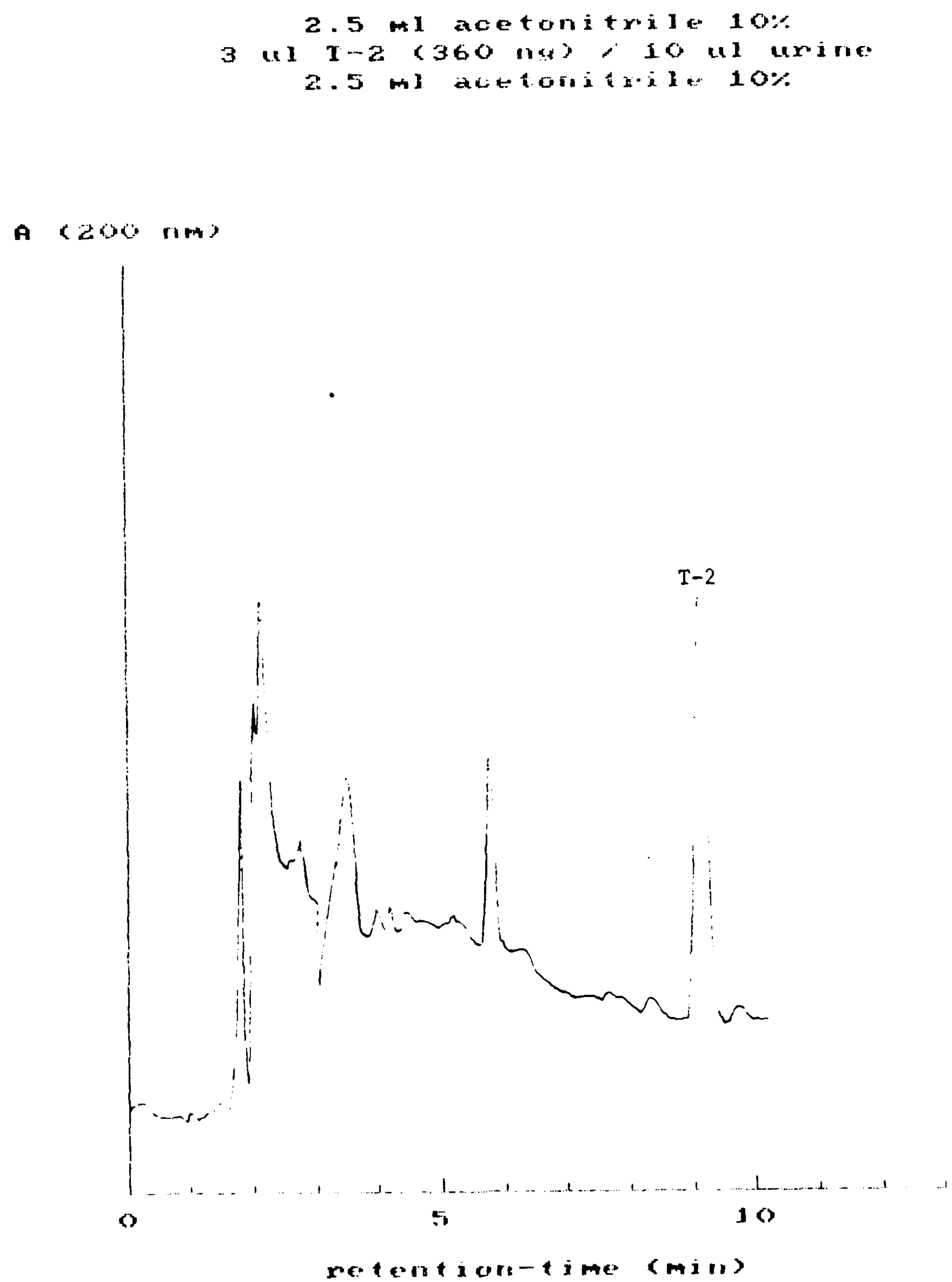

Figure 15 - Recovery of T-2 toxin from urine using pre-column extraction.

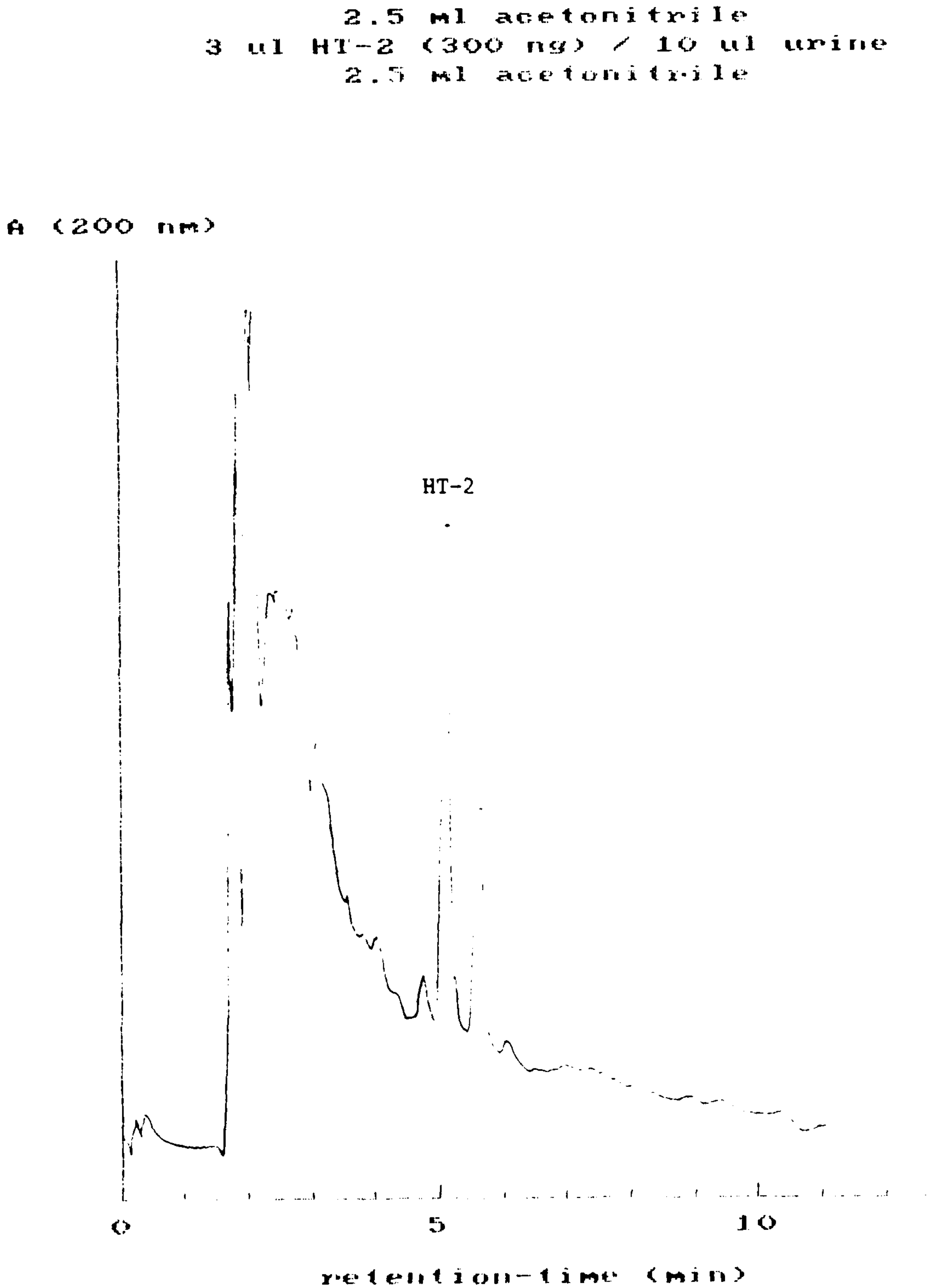

Figure 16 - Recovery of HT-2 toxin from urine using pre-column extraction.

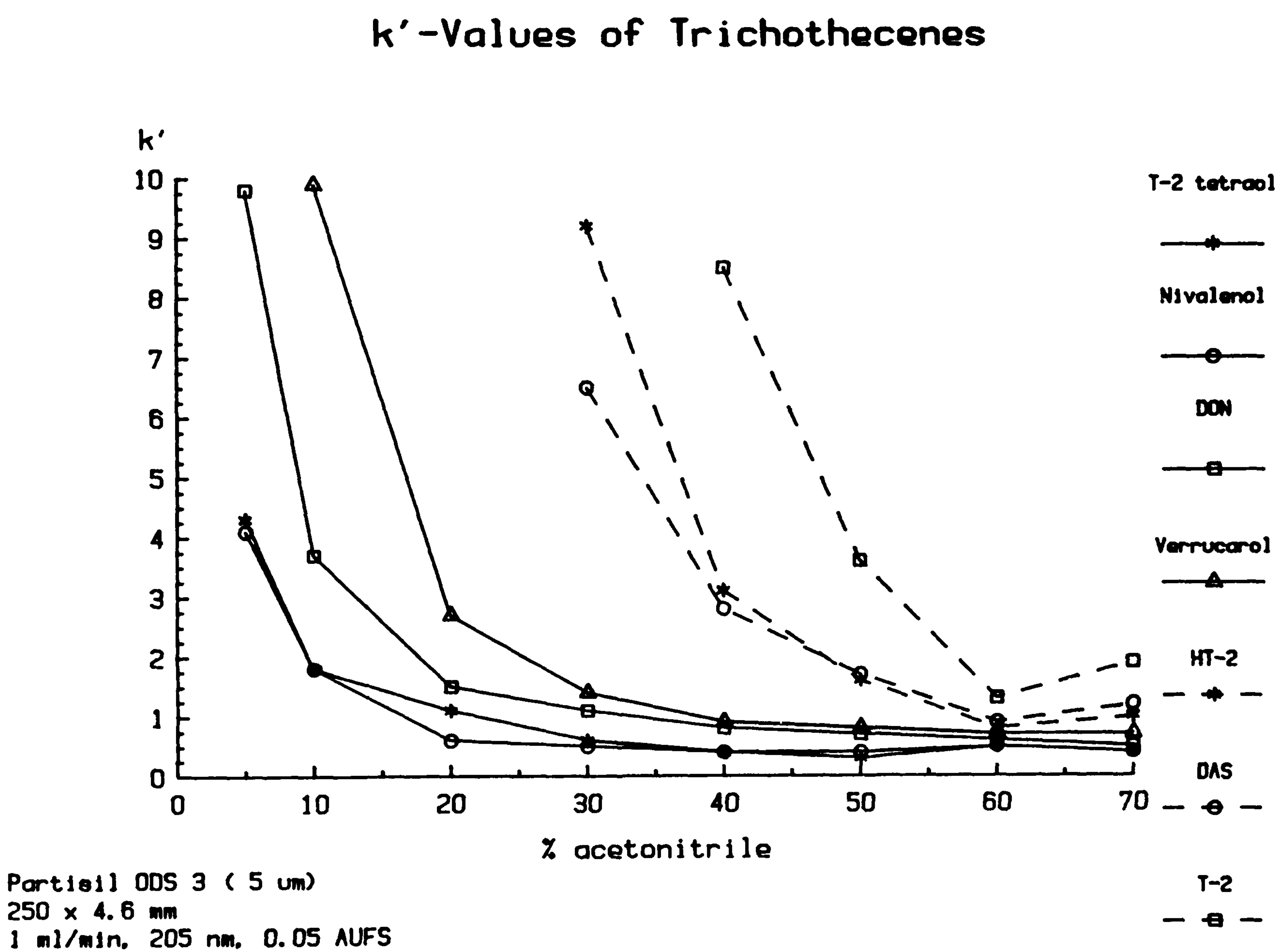

Figure 17 - k' - Values of trichothecenes.

APPENDIX A

THE SYNTHESIS OF DEUTERIUM LABELLED T-2 TOXIN

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SUMMARY

We report the synthesis of isotopically pure trideuteriated T-2 toxin, a useful internal standard for mycotoxin analysis. Peracetylation of HT-2 toxin with hexadeuteriated acetic anhydride, followed by selective hydrolysis of the C-3 acetate afforded the desired compound in good yield.

Key words: T-2 toxin, deuterium, mycotoxin, mass spectrometry, trichothecene.

INTRODUCTION

Trichothecene mycotoxins are a group of sesquiterpenoid compounds produced by several genera of Fungi Imperfecti. Many trichothecenes are known and several of them are natural contaminants of cereal grains(1). Ingestion of the contaminated foodstuffs by humans or farm animals is known to cause a wide variety of toxicoses often leading to death(2). Trichothecenes have also recently been implicated as chemical warfare agents(3).

The determination of trichothecene mycotoxins has been a subject of considerable investigation in recent years(4). The methods used include TLC, GC, GC-MS, LC, and LC-MS(5). Many of these methods require extensive sample preparation to remove the matrix components. In addition, because many of the trichothecenes of interest are very polar and have no chromophoric group for UV or fluorescence detection, derivatization is often needed for quantitative determination(6).

In our analysis of trichothecene mycotoxins and their metabolites in biological fluids by combined HPLC-MS, we required a standard for quantitation. Many standards have been used in GC and MS analyses of trichothecenes including n-dotriacontane(7), known quantities of the analyte(8), and trichothecenes derivatized with deuteriated trimethylsilyl groups(9). However, the ideal internal standard would be a simple

deuteriated sample of the trichothecene to be analyzed. It is therefore surprising that simple deuteriated trichothecenes are not readily available for use as internal standards(10).

There are different approaches to the synthesis of deuterium labelled analogs of trichothecenes, such as T-2 toxin. Previously mentioned was the formation of deuteriated TMS derivatives. The major disadvantage to this method is the widely differing chromatographic behavior between the internal standard and the analyte. Moreover, trimethylsilyl ethers are susceptible to hydrolysis and, in a mixture of TMS-d_0 and TMS-d_9 derivatives of an analyte, cross silylation which may lead to erroneous results is always a possibility. Another approach is to go through an oxidation-reduction sequence as described in the synthesis of radiolabelled T-2 toxin(11). However, this approach is low-yielding and the resultant molecular ion in the mass spectrum of the standard would differ from that of the unlabelled compound by only one mass unit.

In our approach the trichothecene of interest, T-2 toxin, was labelled with deuteriated acetate. Investigations into the chemistry of trichothecenes have shown that the ester groups located in different areas of the molecule exhibit different hydrolysis rates(12). Thus several trichothecenes may be made available by carefully monitored hydrolysis of a per-esterified precursor.

RESULTS AND DISCUSSION

For the synthesis of the title compound 3, we envisioned a two-step sequence (figure 1) involving peracetylation of HT-2 toxin 1 with deuterium

labelled acetic anhydride followed by selective hydrolysis of the C-3 acetate. HT-2 toxin was chosen as the starting material for its ready availability. Acetylation of HT-2 toxin with acetic anhydride-d_6 in pyridine yielded quantitatively the peracetate 2 as evidenced by TLC, the incorporation of six deuterium atoms in the mass spectrum (figure 2), and the presence of only one acetate methyl and the shift of the C-3 and C-4 protons from 4.26 and 4.42 ppm to 5.21 and 5.95 ppm respectively in the NMR spectrum. A portion of peracetate 2 was hydrolyzed in 1M ammonium hydroxide in methanol-water (4:1). After a reaction time of 21 hrs, TLC revealed that very little T-2 toxin 3 was produced. The peracetate 2 extensively hydrolyzed to the starting material, HT-2 toxin, and to more polar products. This result was surprising since Katsonis and Ellison(13), in their investigation of HT-2 toxin formation in liquid culture, prepared hexadeuterio-T-2 toxin in 23% overall yield by a similar procedure. The hydrolysis of peracetate 2 was repeated with careful monitoring by TLC at half hour intervals. After 90 min, TLC revealed that the peracetate 2 was no longer present, and the major component was T-2 toxin 3 along with smaller amounts of HT-2 toxin. The ratio of T-2 toxin to HT-2 toxin in the crude reaction mixture was 4:1 as determined by respective signal heights of the H-2 and H-13 protons in the region 2.5-4.0 ppm of the NMR spectrum. Thus, the overall yield of d_3-T-2 toxin synthesized by this method is nearly 80%, far better than the 23% overall yield reported by Katsonis and Ellison(13) for their d_6-analog. The d_3-T-2 toxin 3, purified by prep TLC, had a mass spectrum (figure 3) identical to the unlabelled compound with the exception of the higher mass peaks which differed by three mass units (m/z 385/382, 367/364, 307/304, 281/278). The isotopic purity for compounds 2 and 3 was determined by mass spectrometry to be greater than 99%.

EXPERIMENTAL

All chemicals were used as obtained from the manufacturer except where noted otherwise. Melting points were obtained on a Thomas Hoover melting point apparatus and are uncorrected. NMR spectra were recorded in deuteriochloroform using a Varian 300MHz spectrometer with tetramethylsilane as the internal reference. Mass spectra were obtained with a Finnigan Model 4000 spectrometer or with a Nuclide Model 1290-G mass spectrometer.

Hexadeuterioacetyl T-2 toxin, 2

HT-2 toxin (Myco Labs, 50 mg) was dissolved in freshly distilled pyridine (1 mL) and hexadeuterioacetic anhydride (1 mL) and allowed to react at room temperature for 24 hrs. Water was then added and the mixture was extracted with ethyl acetate. The organic layer was washed with 1N HCl, water and then dried over sodium sulfate. A pale yellow oil was obtained, 60 mg. NMR:δ 0.77(s, 3H, H-14), 0.98(d, 3H, J = 6Hz, H-4'), 0.99(d, 3H, J = 6Hz, H-4'), 1.77(s, 3H, H-16), 1.92(d, 1H, J = 15Hz, H-7a), 2.10(s, 3H, OAc), 2.39(dd, 1H, J = 5, 15Hz, H-7b), 2.85(d, 1H, J = 4Hz, H-13a), 3.10(d, 1H, J = 4Hz, H-13b), 3.89(d, 1H, J = 5Hz, H-2), 4.14(d, 1H, J = 12Hz, H-15a), 4.24(d, 1H, J = 6Hz, H-11), 4.38(d, 1H, J = 12Hz, H-15b), 5.21(dd, 1H, J = 5, 4Hz, H-3), 5.32(d, 1H, J = 5Hz, H-8), 5.78(d, 1H, J = 6Hz, H-10), 5.95(d, 1H, J = 4Hz, H-4); mass spectrum m/z (rel. intensity) 430(8,M^+-C_4H_8CO), 412(37,M^+-C_4H_9COOH), 370(5), 353(5), 352(6), 326(6), 275(28), 227(44), 180(43), 121(47), 105(52), 85(44), 57(62,$C_4H_9^+$), 46(100,CD_3CO^+).

Trideuterio T-2 toxin, 3

Hexadeuterioacetyl T-2 toxin, 2, was dissolved in a solution (4 mL) of 1M ammonia in methanol-water (4:1). Aliquots of the reaction mixture were analyzed by TLC at 30 min intervals. The TLC plates (Analtech Uniplates silica gel GF) were developed in hexane; ethyl acetate (1:5) and visualized by dipping the plates in a solution of 3% p-nitrobenzyl pyridine in chloroform/carbon tetrachloride (2:3), heating for 30 min at 160°C and dipping in 10% solution of 1,6-hexanediamine in $CHCl_3$-CCl_4 (2:3). The trichothecenes appeared as blue spots on a white background. After 90 min of reaction time the reaction was quenched with 1N HCl and the mixture was extracted with ethyl acetate, washed with water, and dried over sodium sulfate. NMR and TLC analysis indicated that the crude mixture consisted of ~80% T-2 toxin, ~20% HT-2 toxin, and only a trace of starting material. The T-2 toxin residue was purified by TLC (Analtech Uniplates Silica Gel GF) using hexane: ethyl acetate (1:5). The zone corresponding to R_f 0.44 to 0.63 was scraped, and the silica was extracted with ethyl acetate, filtered, and dried (34 mg, 62% isolated yield) mp 144-146°. NMR δ 0.83 (s, 3H, H-

14), 0.99 (d, 3H, J = 7Hz, H-4'), 1.00 (d, 3H, J = 7Hz, H-4'), 1.78(s, 3H, H-16), 1.94(d, 1H, J = 16 Hz, H-7a), 2.07(s, 3H, OAc), 2.43(dd, 1H, J = 16, 6 Hz, H-7b), 2.83(d, 1H, J = 4Hz, H-13a), 3.09(d, 1H, J = 4Hz, H-13b), 3.73 (d, 1H, J = 5Hz, H-2), 4.09(d, 1H, J = 14Hz, H-15a), 4.19(dd, 1H, J = 5, 4Hz, H-3), 4.32(d, 1H, J = 14Hz, H-15b), 4.38(d, 1H, J = 5Hz, H-11), 5.32 (d, 1H, J = 5Hz, H-8), 5.35(d, 1H, J = 4Hz, H-4), 5.84(d, 1H, J = 5Hz, H-10); mass spectrum m/z 385(1.1, $M^+-C_4H_8CO$), 367(3.3, $M^+-C_4H_9COOH$), 325(0.5), 308(2), 307(2), 281(4), 261(2), 205(6), 180(16), 121(51), 105(26), 85(33), 57(69, $C_4H_9^+$), 46(100, CD_3CO^+).

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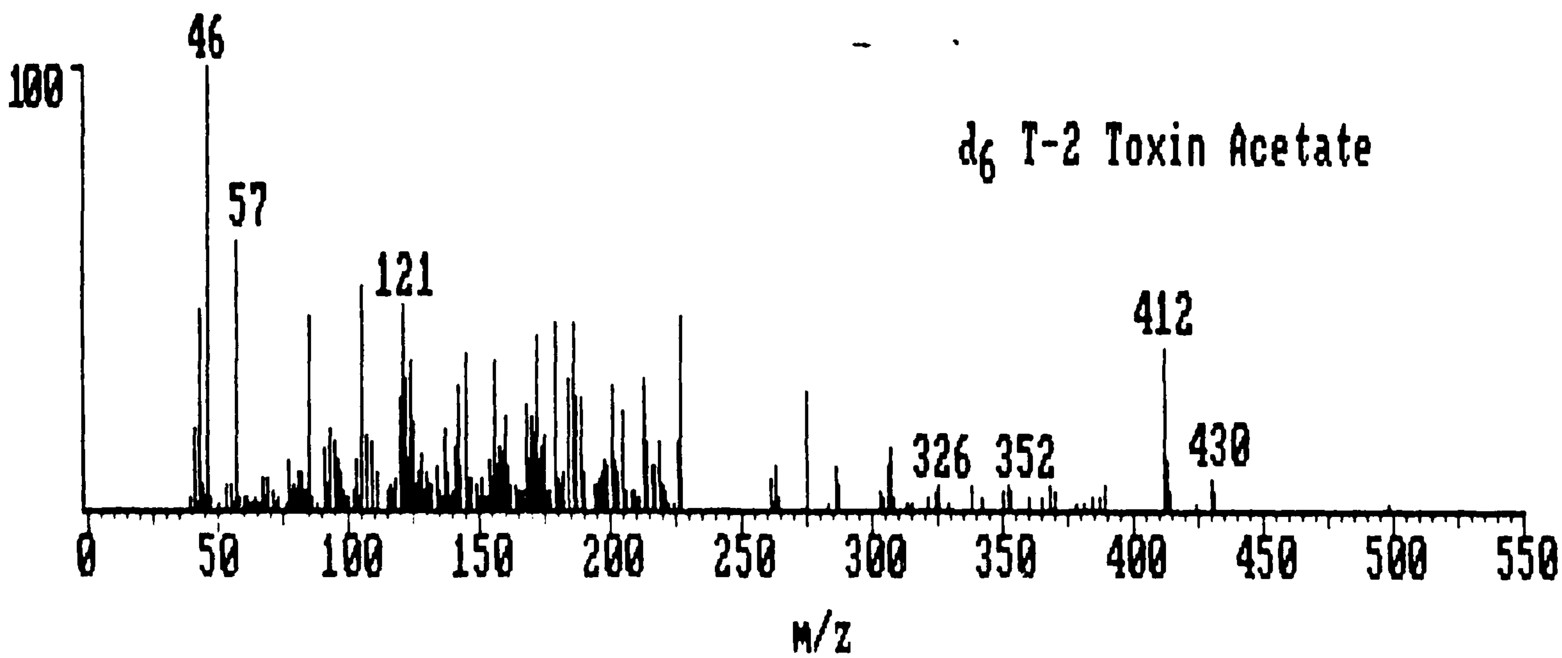
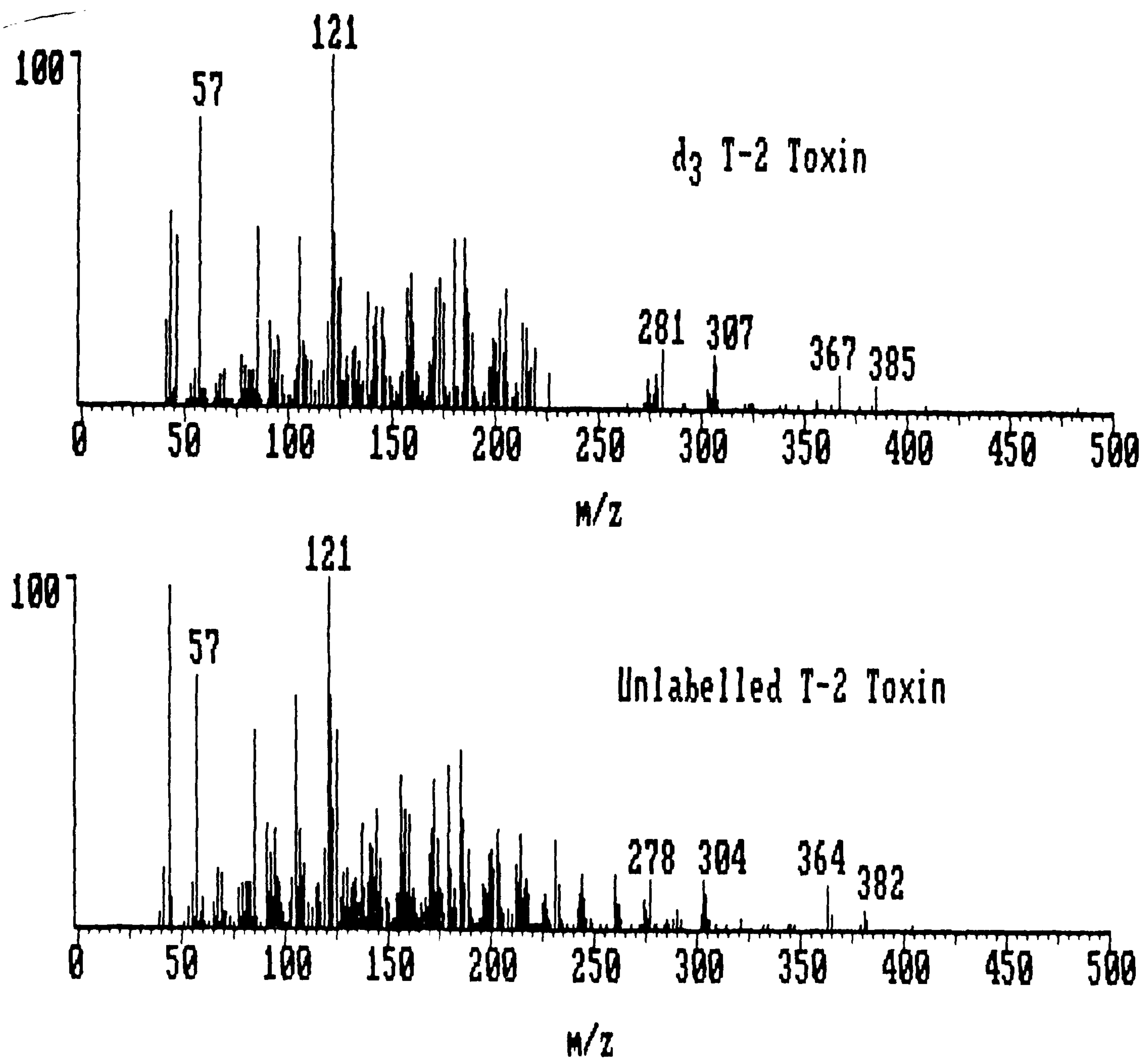


Appendix A, Figure 2 - Mass spectrum of d_6-T-2 toxin acetate.



Appendix A, Figure 3 - Mass spectra of labelled and unlabelled T-2 toxin.

APPENDIX B

Research Plans

The work completed during the first year has effectively laid the foundation for the future course of the program. As is typical of new programs, considerable effort and time were expended on organizational tasks such as personnel hiring and ordering of essential supplies, reagents, etc. We opted during the first year to approach the problem of HPLC/MS analysis of selected trichothecenes from urine in a systematic way so as to avoid any future ambiguities regarding the validity of the methodology. As a result, we are now in a strong position to proceed with efficiency to the next phase of the project, namely the actual HPLC/MS work. To that effect, we intend to commit our newly acquired - with University funds - VG-7070SE mass spectrometer to this project. This instrument is equipped with a moving belt interface and will be used in addition to the Finnigan 4000 MS which has both moving belt and thermospray LC/MS interfaces.

According to the mission of the program, the compounds targetted for analysis by HPLC/MS are T-2, Nivalenol, HT-2, T-2 tetraol, DON, DAS and MAS. The following tasks are projected for completion during the second year continuation of the program:

1. Analysis of Urine Samples

   Analysis of the trichothecenes in urine samples, spiked with the analytes. Initially T-2 will be evaluated since we already have a deuterium labeled analog for use as an internal standard. The other compounds will be quantified using an external calibration curve. On the basis of our work to this date, the concurrent analysis of all analytes from a single HPLC/MS chromatogram should be possible. The limits of detection will be determined and the reliability of the assays statistically evaluated.

2. Labelled Internal Standards

For reliable quantitation of the other analytes, availability of isotopically labeled analogs is necessary. Their need becomes particularly acute at the trace level where reproducibility is often poor. Accordingly, we intend to undertake the synthesis of such analogs as we have done with T-2 itself. In several cases, deuterium or O-18 isotopes can be readily incorporated into the trichothecene nucleus to produce the derived compounds using appropriate starting materials. For example, relatively simple reaction schemes such as those shown in Figures 1 and 2 of this Appendix are envisioned for the production of several labeled hydrolysis by-products of T-2.

3. Analysis of Plasma Samples

Extraction and pre-column cleanup procedures for analysis of trichothecenes in plasma will be evaluated using basic techniques as outlined for urine in the report above. Enrichment from plasma and the study of toxin-protein binding factors by means of HPLC/MS could give new information on the toxicity of these substances. Feasibility studies along these lines appear in order.

4. Analysis of Samples Supplied by the Army

It is our hope that around the fall of 1986 we will carry out analyses of "blind" samples supplied by Ft. Detrick (or other relevant samples suspected of containing the indicated trichothecenes) in order to assess the general applicability of the assays developed here.

5. HPLC/FAB-MS of Trichothecene Conjugates

Up to this point the central focus of our work has been on the analysis of T-2 and its potential metabolites in urine. The metabolic pathways which have been taken into consideration are

those that involve deacylation hydroxylation and de-epoxidation to yield, for the most part, compounds of increased polarity. Recently the isolation of a glucuronide of anguidine established conjugation as an additional pathway for trichothecene metabolism [Rousch, et al., JACS, 107, 3354 (1985)]. This development warrants consideration of alternative analytical approaches for the characterization of these types of metabolites. We refer here to the use of on-line HPLC/MS using fast atom bombardment (FAB) ionization or, of course, thermospray HPLC/MS. In fact, the characterization of such conjugates provides an excellent opportunity to assess the relative merits of HPLC/FAB-MS and thermospray HPLC/MS at least as far as this particular application is concerned. Towards these goals we plan to undertake the following tasks:

(a) Synthesize reference samples of glucuronic acid conjugates of T-2 toxin. We anticipate that the conjugates can be synthesized by the following series of steps:

(i) trimethylsilylation of glucuronolactone

(ii) formation of the bromotrimethylsilyl sugar

(iii) coupling of the bromosugar with T-2 toxin and,

(iv) selective removal of the trimethylsilyl groups to yield the T-2 toxin glucuronic acid conjugate.

For the purposes of the work we plan to prepare a small number of conjugates of a few selected trichothecenes which will serve as model compounds for methods evaluation.

(b) Use a reference mixture to develop an optimized HPLC/FAB-MS system, with primary focus on the retention of chromatographic fidelity. Band variances as a function of

sample deposition will probably figure prominently in this evaluation.

(c) Compare the thermospray and HPLC/FAB-MS processes in terms of chromatographic efficiency, the structural information content of the spectra, and sensitivity. These studies present a unique opportunity to establish comprehensive parameters for the HPLC/FAB-MS analysis of larger molecules in general.

5. Chromatographic Systems

The pre-column systems described in the progress report will be employed. However, we have now prepared microbore columns (1-2mm, i.d.) specifically for this work. This will allow us to work at flow rates of 0.2 mL/min or less. In view of the high aqueous content of the mobile phases required for separation of many of the target compounds, the lower flow rates will facilitate the solvent removal process and provide for more efficient operation of the HPLC/MS system <u>via</u> the moving belt interface.

6. Plan of Work

We expect that the assays for the target compounds including the synthesis of the isotopically labelled internal standards, development of calibration data, statistical evaluation of the data, analysis of spiked urine samples, and examination of selected "blind" samples will be a task that will carry through the end of the calendar year. Examination of the efficacy of the methodologies devised for urine towards the analysis of trichothecenes in plasma, will be conducted concurrently, although this will not be a central focus of the work.

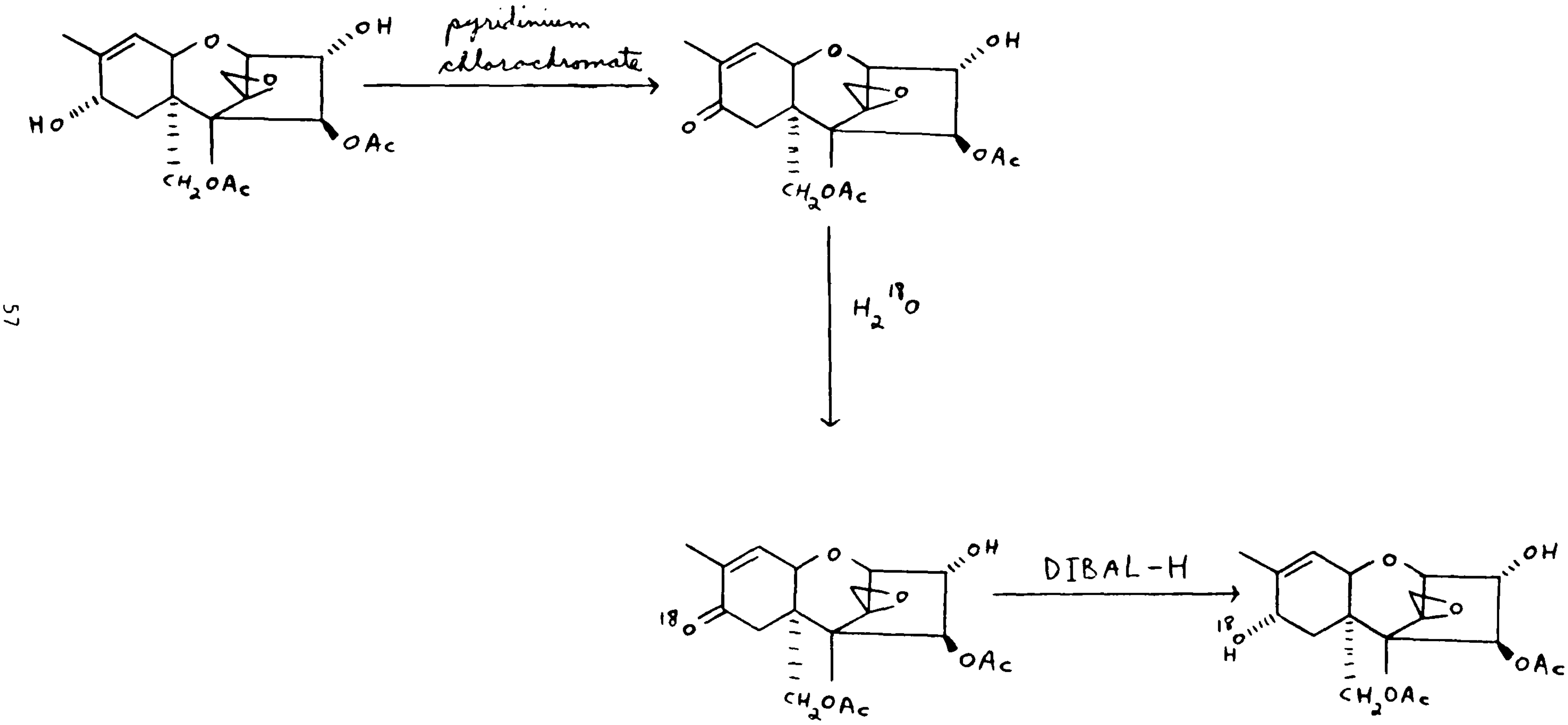
During the middle of the summer we plan to start synthesizing some selected glucuronic acid conjugates. Off-line FAB spectra will be examined initially and then compared to those obtained <u>via</u> the

belt interface. Following determination of the chromatographic conditions for the separation of the conjugates, we will proceed with the optimization of the on-line HPLC/FAB-MS system. Our laboratory is unique in terms of achieving such goals. Nevertheless, completion of these tasks cannot be realistically expected until the end of the second year (Febraury 15, 1987) of the program as originally scheduled.

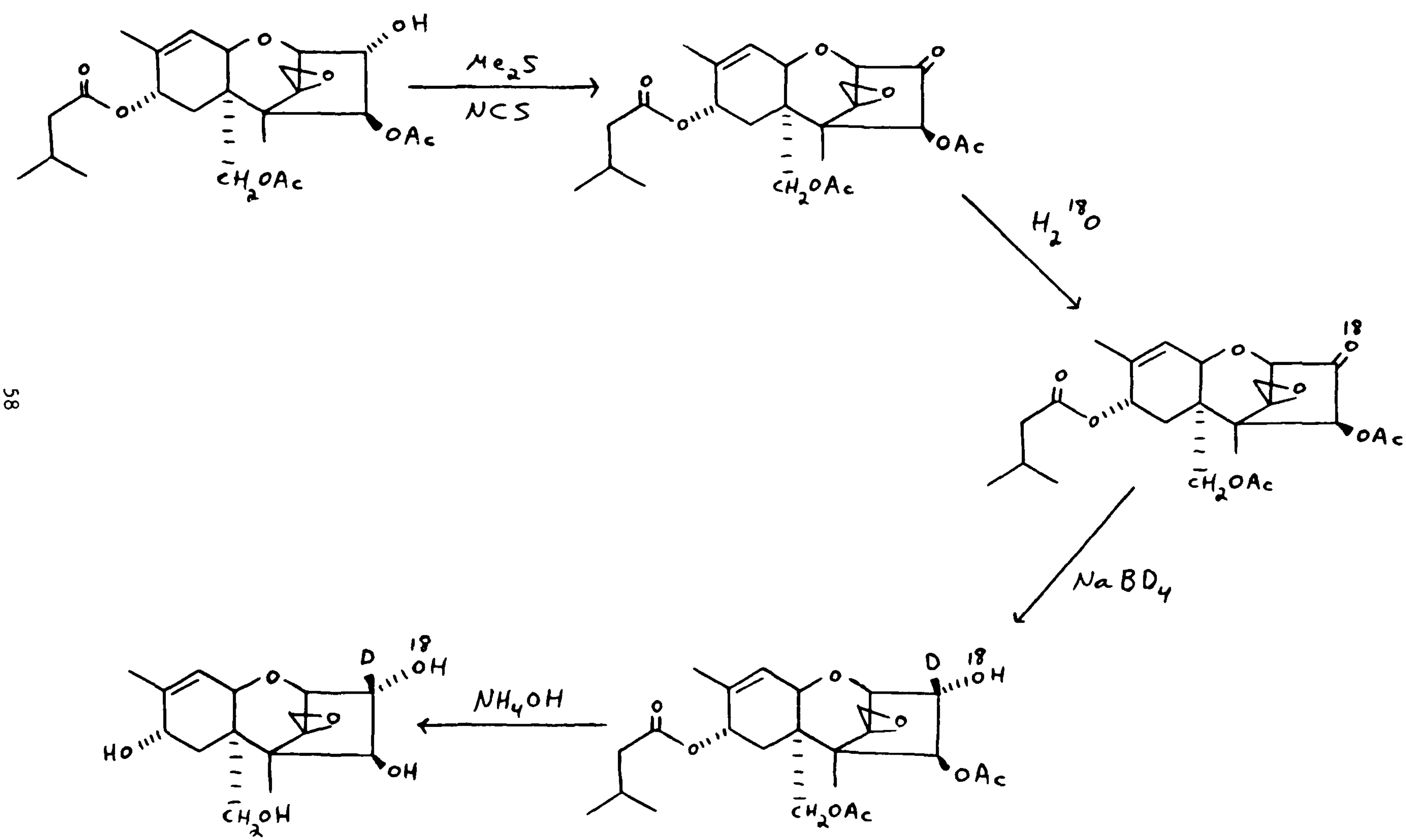
Professors Vouros and Karger will be in charge of the program. Dr. Karger will direct the chromatographic portion of the program. The work will be carried out by Dr. Gerhard Kresback (post doctoral fellow) who joined us on January 1, 1985 from the University of Heidelberg expressly for this project. Dr. Vouros is in charge of the mass spectrometric studies and along with Dr. Dennis Michaud (Institute staff scientist) will also coordinate the synthetic efforts. Dr. Michaud and Mr. Tim Baker (graduate student) will share the responsibilities for operation of the mass spectromter (VG-7070SE and Finnigan 4000).

Personnel Participation for Year I

Dr. Paul Vouros	(15% Academic) (30% Summer)
Dr. Barry L. Karger	(15% Summer)
Dr. Dennis Michaud	(60%, 8/1/85-12/31/85)
Mr. Tim Baker	(50%)
Dr. Gerhard Kresback	(100%, 1/1/86-2/15/86)



Appendix B, Figure 1 - Expected synthetic route to labelled neosolaniol.



Appendix B, Figure 2 - Expected synthetic route to labelled T-2 tetraol.

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